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Využití sacharidových dimerů v imunoterapii nádorů

Carbohydrate dimers in tumor immunotherapy

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V Praze, …… .....……

Podpis

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Abstract

Carbohydrate dimers in tumor immunotherapy

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One possible approach to tumor immunotherapy is an activation of killer lymphocytes through specific ligands for their surface receptors. CD69 is a molecule greatly widespread among various cells of haematopoietic origin. Since the physiological ligand for this receptor is still unknown, ligand mimetics are used for modulation of its activity. The mimetics tested in this work are based on monomeric or oligomeric carbohydrated attached through two different chemical groups to the central linker of varying length, giving rise to thiourea and triazole series. The ability to precipitate soluble NKR-P1 and CD69 receptors was evaluated in precipitation assays and the optimal length of the linker for NKR-P1 receptor was found to be decyl. On the other hand, cross-linking of CD69 is not so dependent on the length of the linker.

The aim of this work was to describe *in vitro* effect of the tested compounds on cellular signalization, natural killing of leukemic cell lines and activation-induced apoptosis. Compounds of triazole series containing two disaccharides (GalNAc β1→4 GlcNAc) linked by a linker were found to have the strongest effect on the production of inositolphosphates and the elevation of intracellular calcium ions, as well as on the natural killing. Although compounds belonging to the triazole series showed almost none effect on activation-induced apoptosis, the compounds of thiourea series affected greatly cytotoxicity-induced cell death. The activation abilities of the tested carbohydrate dimers make them suitable for evaluation in experimental *in vivo* animal tumor therapies. (In English)

Key words: lymphocyte activation, tumor immunotherapy, apoptosis, ligand mimetics, NK cells, CD69.

Abstrakt

Využití sacharidových dimerů v imunoterapii nádorů

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Jednou ze strategií pro imunoterapii nádorů je aktivace cytotoxických lymfocytů pomocí ligandů specifických pro jejich povrchové receptory. CD69 je široce rozšířeným receptorem na povrchu mnoha buněk hematopoietického původu, a protože jeho fyziologický ligand nebyl zatím popsán, na modulaci jeho funkcí se dají použít různá ligandová mimetika. Mimetika testované v předkládané práci jsou založené na mono- či disacharidech spojených pomocí dvou různých funkčních skupin, které tvoří thiomočovinovou a triazolovou sérii. Při precipitačních studiích byla sledována schopnost precipitovat rozpustný NKR-P1 a CD69 receptor, přičemž se zjistilo, že optimalizovaná délka spojky pro navázání látky na NKR-P1 receptor je decyl. Naproti tomu aktivace receptoru CD69 neni až tak závislá na délce spojky.

Cílem této práce bylo z imunologického hlediska prozkoumat vlivy jednotlivých testovaných látek na bunečnou signalizaci, cytotoxicitu vůči buňkám nádorové linie a aktivaci vyvolanou apoptózu. Největší vliv na signalizaci, sledovanou pomocí produkce inositolfosfátů a vápenatých iontů a stejně tak na přirozené zabíjení, měly látky triazolové série obsahující ve své molekule dva disacharidy (GalNAc β1→4 GlcNAc) spojené spojkou. Zatímco látky triazolové serie neměly skoro žádný vliv na apoptózu efektorových buněk vyvolanou aktivací, látky thiomočovinové série způsobovaly aktivací indukovanou buněčnou smrt. Zejména jedna látka z triazolové série vykazovala v imunologických testech právě tak jako v precipitačních studiích s rozpustným lidským CD69 receptorem velmi dobré výsledky. Tyto aktivační schopnosti předurčují testované látky pro *in vivo* testováni na zvířecích modelech. (Práce je psána anglicky)

Klíčová slova: aktivace lymfocytů, imunoterapie nádorů, apoptosa, ligandové mimetiká, NK buňky, CD69.

Content

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AIF	Apoptosis-inducing factor
APC	Antigen presenting cell
ATP	Adenosine triphosphate
CD	Cluster of differentiation
CICD	Cytotoxicity-induced cell death
CRD	Carbohydrate recognition domain
CTL	Cytotoxic T lymphocyte
CTLD	C-type lectin-like domain
DAG	1,2-diacylglycerol
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
EGTA	Ethyleneglycol-*O,O'*-bis(2-aminoethyl)-*N,N,N',N'*--tetraacetic acid
GalNAc	*N*-acetyl-D-galactosamine
GlcNAc	*N*-acetyl-D-glucosamine
GTP	Guanosine triphosphate
HLA	Human leukocyte antigen
ICAM-1	Inter-cellular adhesion molecule 1
IFN-α	Interferon-α
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukine
Ins2P	Inositol-1,4-bisphosphate
Ins3P	Inositol-1,4,5-trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KIR	Killer inhibitory receptor
MALT	Mucous associated lymphoid tissue
MHC	Major histocompatibility complex
MPR	Mannose phosphate receptor

NCRs	Natural cytotoxicity receptors
NKC	Natural killer gene complex
NK cell	Natural killer cell
NKD	Natural killer domain
PBMC	Peripheral blood mononuclear cells
PI3K	Phosphoinositide-3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP4K	Phosphoinositide-4-kinase
PIP5K	Phosphoinositide-5-kinase
PMA	Phorbol myristate acetate
PS	Phosphatidylserine
RNA	Ribonucleic acid
TCR	T cell receptor
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor α

1. INTRODUCTION

1.1. Immune system

An immune system is a highly evolved system of biological structures and molecules whose function is to identify and eliminate foreign material and tumor cells. In order to do this, complex mechanisms have been evolved that distinguish between foreign molecules and the molecules that constitute self./1/ A powerful capability for learning, memory and pattern recognition is necessary for performing this task. Since immune system employs genetic mechanisms to provide variability of biological structures similar to those used in biological evolution and these function on a relatively short time scale (few days), some studies state that the immune system is an ideal candidate for computer modeling of adaptive processes./2/

Responses of the immune system of an organism can be divided into two fundamentally different types. Innate responses occur to the same extent whenever an infectious agent is encountered. They are based mainly on the recognition of strongly evolutionary conserved foreign structures by cellular components (phagocytic cells, cells that release inflammatory mediators such as basophils, eosinophils and mast cells, and natural killer cells) and molecular components of the immune system (mainly complement and cytokines). The second type of immune system responses are called acquired or adaptive responses and they involve the proliferation of antigen-specific B and T cells that occurs when the surface receptors of these cells bind to a specific antigen./3/ Both of these types of immune system responses are composed of cell-mediated and humoral part.

1.1.1. The cells and tissues of immune system

Bone marrow and thymus, usually referred to as primary lymphatic organs, are the place of genesis, differentiation and maturation of immunocytes. Especially bone marrow contains haematopoietic stem cells whose characteristic sign is an expression of CD34 on their surface, that give rise to a variety of leukocytes, erythrocytes and platelets. Spleen, lymphatic nodes, Peyer's patches and mucous associated lymphoid tissue (MALT) are called secondary lymphatic organs. They

are the sites of proliferation and differentiation of effector cells induced by the contact with APC (antigen presenting cells) and therefore play an important role in antigen specific immune responses.[/3/]

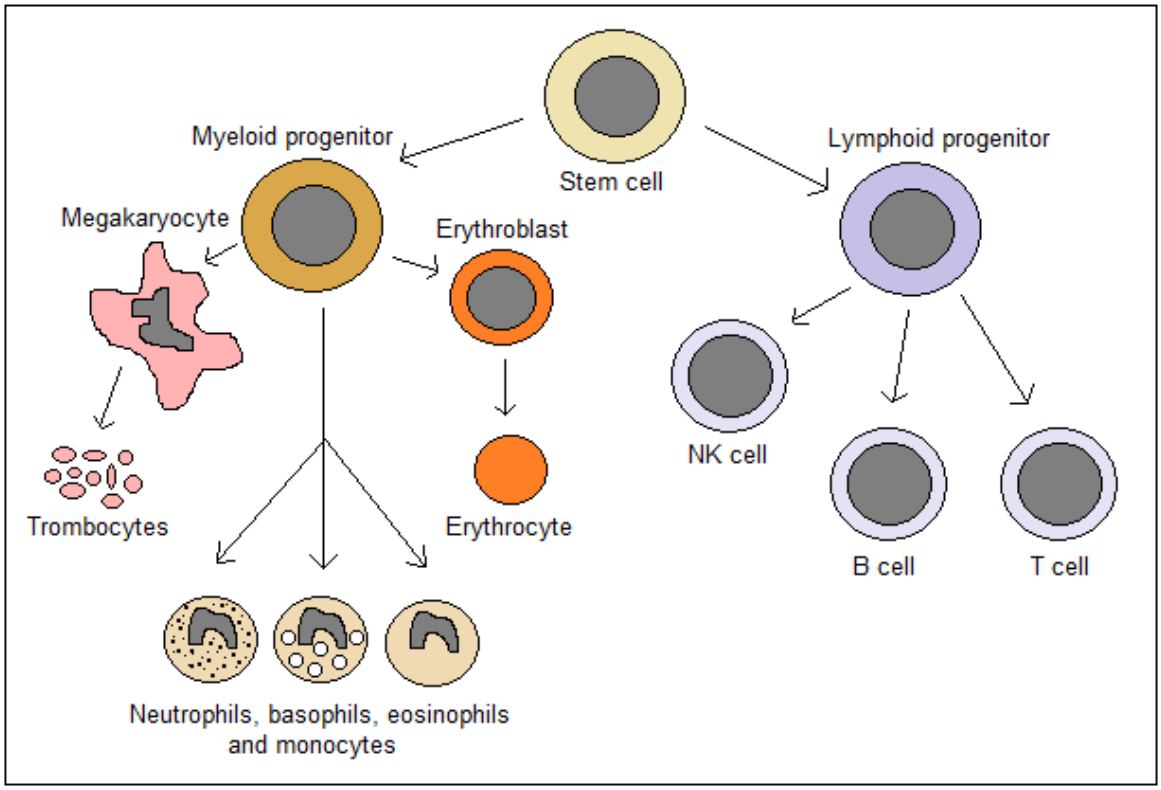

Figure 1. Differentiation of leukocytes from haematopoietic stem cells: *Myeloid lineage gives rise to monocytes that can differentiate to macrophages or dendritic cells, and to three types of granulocytes – neutrophils, eosinophils and basophils. These form an important part of innate immune responses even though dendritic cells and macrophages belong to antigen-presenting cells and therefore participate in adaptive immune responses. Lymphoid lineage gives rise to NK cells, B and T lymphocytes. B and T cells participate in acquired immune responses while NK cells play a crucial role in innate immunity. (Modified according to ref. /1/ and /3/)*

As it was mentioned earlier, haematopoietic stem cells in the bone marrow can differentiate to various types of leukocytes. Progeny of these stem cells progressively lose self-renewal capacity and become restricted to one lineage. Clonogenic common progenitor that can differentiate into T, B and natural killer cells has been identified, complementary clonogenic common myeloid progenitor giving rise to megakaryocyte/erythrocyte or granulocyte/macrophage progenitors is proposed to exist.[/4/] This stem cell differentiation is discussed on Figure 1. As mentioned earlier, an important role in innate immune responses play phagocytic

cells such as macrophages, neutrophils and dendritic cells. These cells are able to engulf foreign particles via a specific form of endocytosis and therefore eliminate various microorganisms or their parts. Other cells participating in innate immune system responses are mast cells, eosinophils, basophils and natural killer cells with diverse functions. Acquired immune responses are carried out by lymphocytes whose major representatives are B cells and T cells.

1.2. Natural killer cells

Natural killer cells can be characterized as lymphocytes mediating innate protective responses against a variety of pathogens and tumor cells. They were originally identified by their ability to spontaneously lyse tumor cell lines, by the lack of TCR and CD3 characteristic for T cells and by their large granular morphology.[5] They comprise up to 15 % of peripheral blood lymphocytes.[6] Most data suggest that thymic processing is not necessary for their functioning and that NK cells are entirely differentiated in the bone marrow. After being released from the marrow, NK cells circulate in peripheral blood or migrate mostly to spleen. Lifespan of NK cells is not well defined since lifespans ranging from a few days to several months have been described in laboratory animals.[7] Interestingly, studies has shown that in humans, NK cells can be divided into two subsets according to their cell-surface density of CD56. $CD56^+$ subset has lower cytotoxicity effects in comparison with $CD56^-$ subset which expresses higher levels of Ig-like NK receptors.[8] However $CD56^+$ subset of natural killer cells provides higher production of regulatory molecules such as IFN-γ, TNF-α, IL-10 or IL-13.[5]

1.2.1. Role of NK cells in immune system

NK cells maintain several different functions depending on the expression of different receptors on their surface, for example production of cytokines linked to the regulation of adaptive immune responses and the most notably, spontaneous non-MHC-restricted cytotoxicity that is a very useful tool for elimination of tumor or virus-infected cells. In principle, altered cells should be recognized by T cell receptors whose ligands are small peptide fragments presented by MHC-I (analogous HLA class I in humans). MHC-I displays 8-10 amino acid fragments of

intracellular proteins synthetised by a target cell, digested in proteosomes nad presented via endoplasmic reticulum on cell surface, and therefore in case of viral infection or transformation of a target cell when foreign protein structures are present in the cytoplasm, $CD8^+$ T cells are activated. A common evolutionary adaptation of viruses and tumor cells for escaping the cytotoxic reaction of CTLs is a downregulation in expression of MHC-I.[9] This loss of HLA class I antigens on cell surface was described particularly in case of the breast cancer, skin cancer, prostate cancer and lung cancer.[10, 11, 12]

Since MHC-I is often downregulated as a result of cellular transformation or viral infection, specific mechanisms have been evolved in NK cells that enable them monitor the quantitave expression of these cell surface molecules and induce a cytotoxic reaction towards so identified target cells.[13] So called *missing-self recognition* mechanism proposes that MHC-I expressed on all nucleated cells is recognized by various inhibitory receptors of NK cells, that upon binding to their ligands block the cytotoxic activity of an effector cell.[14] Therefore cytolysis is inhibited when appropriate MHC-I molecules are present on the surface of the target cell, and it is enhanced when target cells are deficient in MHC-I.[13] Integration of signals from inhibitory and numerous activating receptors present at the surface of NK cell occurs and can possibly lead to initiation of cytotoxic reaction. Besides the spontaneous antibody-independent cytotoxicity, NK cells can mediate antibody-dependent cellular cytotoxicity (ADCC). In ADCC, antibodies coating the target cell are recognized by Fc receptors present at the surface of effector cell upon which activation a target cell is lysed. Unlike antibody-independent cytotoxicity, NK cells share this capability with monocytes and granulocytes.[7]

1.2.2. Mechanism of NK cell-mediated cytotoxicity

The main principle of immune response involves the specific recognition of an antigen by responding immunocyte. To fulfill this task, specific structures must be expressed on the cell surface of an immunocyte that can interact with antigens. Upon ligand binding to a specific receptor, activation of receptor-linked signal transduction pathways occurs.[15]

When NK cell is activated after the interaction of its activating receptors with ligands, the phosphoinositide signaling cascade occurs. Phosphatidylinositol-

4,5-bisphosphate (PIP_2) which accounts for less than 0.05 % of phospholipids constituting a cytoplasmic membrane, is upon effector cell activation hydrolyzed by phospholipase C to inositol-1,4,5-trisphosphate (Ins3P) and 1,2-diacylglycerol (DAG)[16]. Both of these molecules then act as second messengers. While DAG remains associated with the membrane, water-soluble Ins3P diffuse from the site of activation and upon its presence in the cytoplasm of NK cell, Ca^{2+} ions are released from intracellular stores (calcium efflux) followed by a more prolonged phase of calcium entry from extracellular space (calcium influx). Calcium efflux is explained by an interaction of Ins3P with a specific membrane-bound receptor localized on endoplasmic reticulum that opens Ca^{2+} channel closely associated with the receptor. The size of intracellular Ca^{2+} pool is believed to be regulated by a GTP-dependent mechanism.[16] Inositol-1,4,5-trisphosphate is then dephosphorylated by a specific phosphoinositide-5-phosphatase to inositol-1,4-bisphosphate.

Phosphatidylinositol has 5 free hydroxyl groups that can be phosphorylated even though only those in 3-, 4- and 5-position are known to be phosphorylated *in vivo*. Interchange between the molecules phosphorylated in different positions is catalyzed by a group of ATP-dependent enzymes known as phosphoinositide-3-kinases (PI3Ks), phosphoinositide-4-kinases (PIP4Ks) and phosphoinositide-5-kinases (PIP5Ks). Their various products function as second messengers and control a wide range of processes such as cell survival, cytoskeletal rearrangement, proliferation and mainly vesicle trafficking in secretory and endocytic pathways.[17] Many studies suggest that especially PI3K makes a highly regulated spot in NK cell-mediated cytotoxicity.[18]

Due to these molecular changes, cytokines such as TNF-α and IFN-γ that regulate inflamatory and anti-tumor responses are produced and cytotoxic granules containing mediators of cell-death pathways (mostly noted perforin and granzymes) are released into the contact synapse with target cell by exocytosis. Granzymes are necessary for triggering apoptosis in target cells, but they depend on being appropriately delivered by perforin through a mechanism that is still under discussion. This statement is supported by the evidence showing that perforin-deficient mice generated by homologous recombination failed to lyse infected and tumor cells.[19] Some possible mechanisms for the delivery of granzymes into a target cell are discussed on Figure 2. Although an accurate mechanism by which

these molecules induce apoptosis in target cells is unclear, it is thought to include the cleavage of pro-caspase proteins by granzyme B in caspase-mediated cell death, or to trigger caspase activation indirectly through the activation of specific proteins resulting in a leakage of pro-apoptotic mitochondrial mediators such as cytochrome *c*.[20] Synthesis of various cytokines or surface activation antigens and possible proliferation follows up the activation of an effector cell.[21]

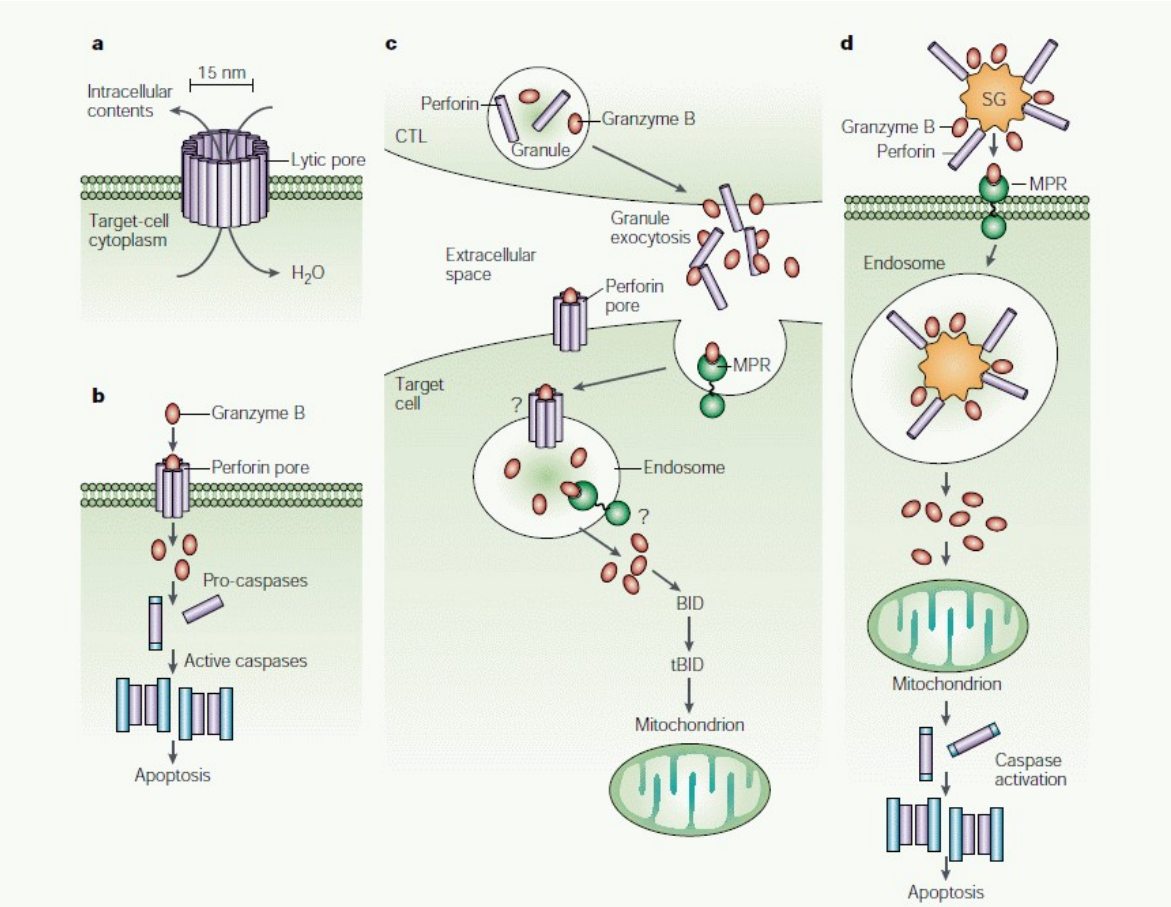

Figure 2. Evolving models for NK-cell-induced cell death[20]: ***(a)*** *First model introduced statement that perforin acts as a lytic molecule forming pores in the cytoplasmic membrane of a target cell what then results in the loss of plasma-membrane homeostasis.* ***(b)*** *After the discovery of granzymes and their role in NK-cell-induced cell death, it was proposed that perforin forms pores in the cytoplasmic membrane of a target cell by which granzymes can enter via passive diffusion and cause the activation of caspases.* ***(c)*** *The discovery that granzymes enter target cells by endocytosis independently of perforin lead to the model stating that perforin and granzymes enter target cells by endocytosis and pores made by perforin are used by granzyme molecules to leak out from endocytic vesicles.* ***(d)*** *The latest and the most discussed model proposes that granzyme B is a ligand for MPR upon whose activation a macromolecular complex containing perforin, granzymes and possibly other molecules forms and can be taken up into target cells without significant perforin-pore formation.*[20]

1.2.3. Receptors of NK cells

Natural killer cells and their functions are regulated by a range of inhibitory and activating receptors. The inhibitory receptors are often dominant on the cell surface, which initially obscured the activity of the activating structures. There is not a single antigen receptor responsible for the activation of NK cells. Instead, the concerted activity of several activating or inhibiting receptors triggers NK cell effector function.[22] Ligands such as sialic acid, collagen or MHC were described to bind to different receptors of immunoglobulin, integrin and calcium-dependent families expressed on the surface of NK cells.[23] Activating receptors are often characterized by an immunoreceptor tyrosine-based activation motif (ITAM) while inhibitory receptors in many cases exhibit immunoreceptor tyrosine-based inhibition motif (ITIM).[22] Consensus sequence of ITAM/ITIM motif is shown on figure 3. These ITAM and ITIM modules possess conserved tyrosine residues that undergo rapid phosphorylation upon receptor ligation. As a result, an interaction of protein tyrosine phosphatases SHP-1 and SHP-2 with the phosphorylated receptors occurs and by this mechanism, the key components of the signaling cascade in effector cells are regulated.[14, 15]

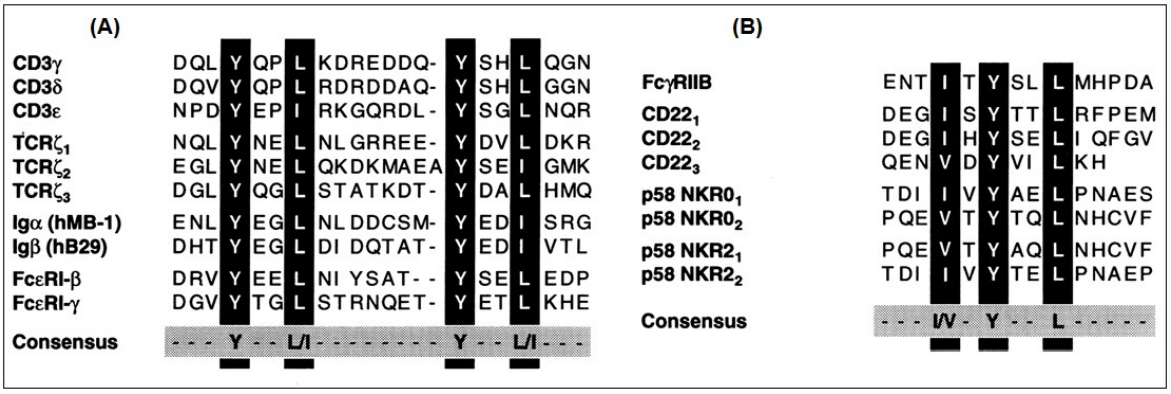

Figure 3. Several activating and inhibitory receptors exhibiting ITAM or ITIM sequence[15]: (A) *A partial amino acid sequence of several activating receptors with ITAM motif;* ***(B)*** *a partial amino acid sequence of several inhibitory receptors with ITIM motif. The consensus ITAM sequence consists of a duplication of YxxL/I sequence with several intervening amino acids, consensus ITIM sequence consists of I/VxYxxL sequence.*

A large part of inhibitory receptors expressed by NK cells recognize already mentioned MHC-I and therefore provide protection for cells that express sufficient amounts of these molecules on their surface. The inhibitory isoforms of the human

killer cell immunoglobulin-like receptors (KIR) recognizing polymorphic HLA class I serve as an example.[22, 24] Other examples of inhibitory NK-cell receptors include Ly-49, NKG-2A and NKR-P1[25], which by contrast is recognized as an activating receptor in rats[26]. NK cell activation is trigerred by activation receptors whose eminent part form natural cytotoxicity receptors (NCRs). Although they belong to the immunoglobulin superfamily, they exhibit only a negligible homology with known human cell-surface molecules or with each other. Their function is normally downregulated by coaggregation with KIRs, and thus the induction of NK-cell activation via NCRs is possible only if the KIR-HLA interaction is absent.[25] NKG-2D, CD44, CD69 and CD2 may serve as other examples of NK-cell activating receptors.

1.3. CD69 antigen

CD69 belongs to the type II integral membrane proteins with a C-type lectin-like domain. It is encoded by a gene located on chromosome 12 in humans.[27] Since this genetic region contains genes that encode several receptors expressed primarily on NK cells, it is often referred to as NK gene complex (NKC). Members of individual NKC gene families show more than 70% identity to each other at the nucleotide level, even though there is less than 3% nucleotide-level identity between different families. Besides CD69 gene, NKC bears genetic material encoding for example NKR-P1, NKG2 and CD94.[28]

CD69 belongs to the first molecules expressed on the surface of lymphocytes early following their activation. Its expression is enhanced by different stimuli such as PMA, IL-2, IL-12, IFN-α or anti-CD16 monoclonal antibodies.[29] However, its expression is not restricted only to activated lymphocytes, as its constitutive expression was described on platelets, bone marrow myeloid precursors, activated neutrophils and eosinophils. More interestingly, CD69 has been found also on circulating monocytes, epidermal Langerhans cells and a small percentage of resident lymphocytes in thymus and secondary lymphoid tissues.[30, 31] Such a wide cellular distribution, along with its signal-transducing properties proved in all of these cell types, indicates an important role for CD69 receptor in the biology of hematopoietic cells.[31, 27]

1.3.1. Structure of CD69

Structurally, human CD69 is a homodimer formed by the association of 28 kDa and 32 kDa chains held together by disulfide bridges. Cloning and expression of human CD69 gene has revealed that the two chains present in the protein result from the differential glycosylation of a 22.5kDa polypeptide encoded by a single gene.[30a] This polypeptide has 199 amino acids in length, of which approximately 40 form the intracellular portion of the molecule, followed by 21-residue transmembrane region and an extracellular region referred to as NKD of 118 amino acids.[31] There are three disulfide bonds in CD69 NKD. The extracellular portion of the molecule contains a C-type lectin-like domain (CTLD) that is a conserved protein module found in numerous proteins with a wide range of functions. Unlike classic C-type lectin proteins able to bind sugar residues due to a coordination of Ca^{2+} ion (so called CRDs), CTLD fold may serve functions other than only saccharide recognition.[31]

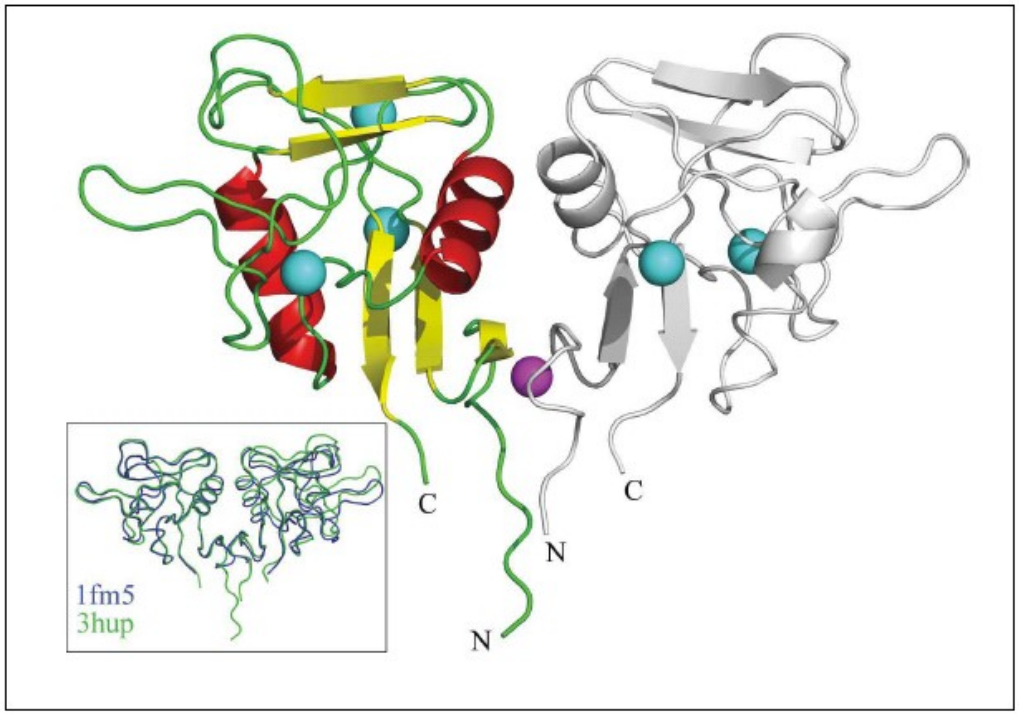

Figure 4. Crystal structure of CD69[32]: *The structure of extracellular domain of human CD69 determined by single-crystal X-ray diffraction is shown.*

Unlike the mouse CD69 with three potential sites for N-linked glycosylation, there is only one potential N-glycosylation site in human CD69 molecule, located at

amino acid position 166. Due to a qualitative heterogenity in chain glycosylation, 28 kDa and 32 kDa glycosylated chains are produced. These products can then together with non-glycosylated monomeric unit randomly assemble into different dimers.[30] Symetric or slightly asymetric binding of these monomers has been described. Both chains are constitutively phosphorylated on serine residues what is thought to have important implications for signal generation.[30] A structure of CD69 molecule is shown in Figure 4 (page 18).

1.3.2. Biological function of CD69

Biological function of a receptor molecule is closely linked to the physiological ligands it can recognize. Since physiological ligands for CD69 receptor are still unknown, specific biological function of this molecule is not yet entirely understood. However, data suggest that CD69 molecule could act as a proinflammatory receptor mediating immune cell activation and participating in inflammatory responses either directly or inderectly.[33] On the other hand, mediation of several inhibitory signals *in vitro* was described and therefore CD69 is viewed more as a co-stimulatory receptor rather than a net inhibitory or activating molecule.[33] A production of TGF-β in $CD4^+$ and $CD8^+$ T cells as well as NK cells upon receptor cross-linking was described[34] and together with regulating other cellular functions such as proliferation, TNF-α production and the expression of other antigens such as CD25 and ICAM-1[29], it is clear that CD69 accounts for a pivotal role in biology of haematopoietic cells.

Several studies have shown that monomers of CD69 are able to ligate calcium atom in a single site formed by Asp 171 and the two adjacent glutamic acids Glu 185 and Glu 187 and that this Ca^{2+} binding results in a conformational change near Thr 107 and Lys 172 residues. These conformational changes lead to the formation of high-affinity binding sites for *N*-acetyl-D-glucosamine, and similarly, a structural change in Glu 185 and Glu 187 creates a high-affinity site for *N*-acetyl-D-galactosamine.[35, 36] It has also been proposed that high affinity ligands would be among the branched oligosaccharides rather than linear structures.[37] Recently, carboxylated calixarenes were identified as a new class of high affinity ligands for CD69 receptor[38] proving that not only saccharide structures can serve as ligands for this receptor. Ability to bind the pentapeptidic sequence LELTE that

is derived from the mycobacterial heat shock protein Hsp65 with high affinity serves as an example of variable binding abilities of CD69. Different conjugates of this pentapeptidic sequence coupled onto a peptidic scaffold have been prepared and their immunological activity has been tested.[39] These experiments proved that multimeric presentation of possible ligands for CD69 antigen results in cross-linking of the receptors present on the surface of effector cell and can cause accentuated cellular response.

1.4. Immune system and cancer

Cancer originates from mutant DNA sequences whose molecular products regulate crucial processes such as cell survival, tissue homeostasis and cell death. A lot of hereditary cancer syndromes originate from mutations in genes that mediate responses to DNA damage, what generates genetic instability. In this state, new antigens characteristic for so altered tissue are constantly being produced, what is in comparison with nontransformed tissue which maintains a stable antigenic profile. In addition, hundreds of genes that are inactive or expressed at a low level in healthy tissues are activated dramatically in cancers, what affects their antigenicity.[40] Collaborative interactions between various cell types such as fibroblasts, epithelial cells, immune cells and cells forming blood and lymphatic vasculature, and the ability of mutant cells to harness these collaborative capabilities is necessary for cancer development.[41]

A very important regulatory role of immune system in carcinogenesis has been proposed almost a hundred years ago. Even though the historical viewpoint suggested that the host immunity is protective with regards to cancer, it is now known that besides this protective role, chronically activated immune cells can promote growth and survival of neoplastic cells in certain conditions.[41]

One approach to describe the protective role of immune system with regards to cancer is earlier mentioned cytotoxic activity of cytotoxic lymphocytes towards the cells presenting peptide fragments of altered proteins through MHC-I. Since the expression of MHC-I on tumor cells can be downregulated, NK cells can provide elimination of so transformed cells by the mechanism of *missing-self recognition*. The other approach regards immune surveillance as protection against certain

pathogen-associated cancers by preventing or regulating the viral infection that can lead to cancer.[40] It is believed that over 15 % of human cancers is caused by infectious agents and inflammatory states an infection leads to.[41] It is now known that incidence of cancer is closely linked to inflammation states mediated by cells of native immune system and that these changes can faciliate tumor growth.

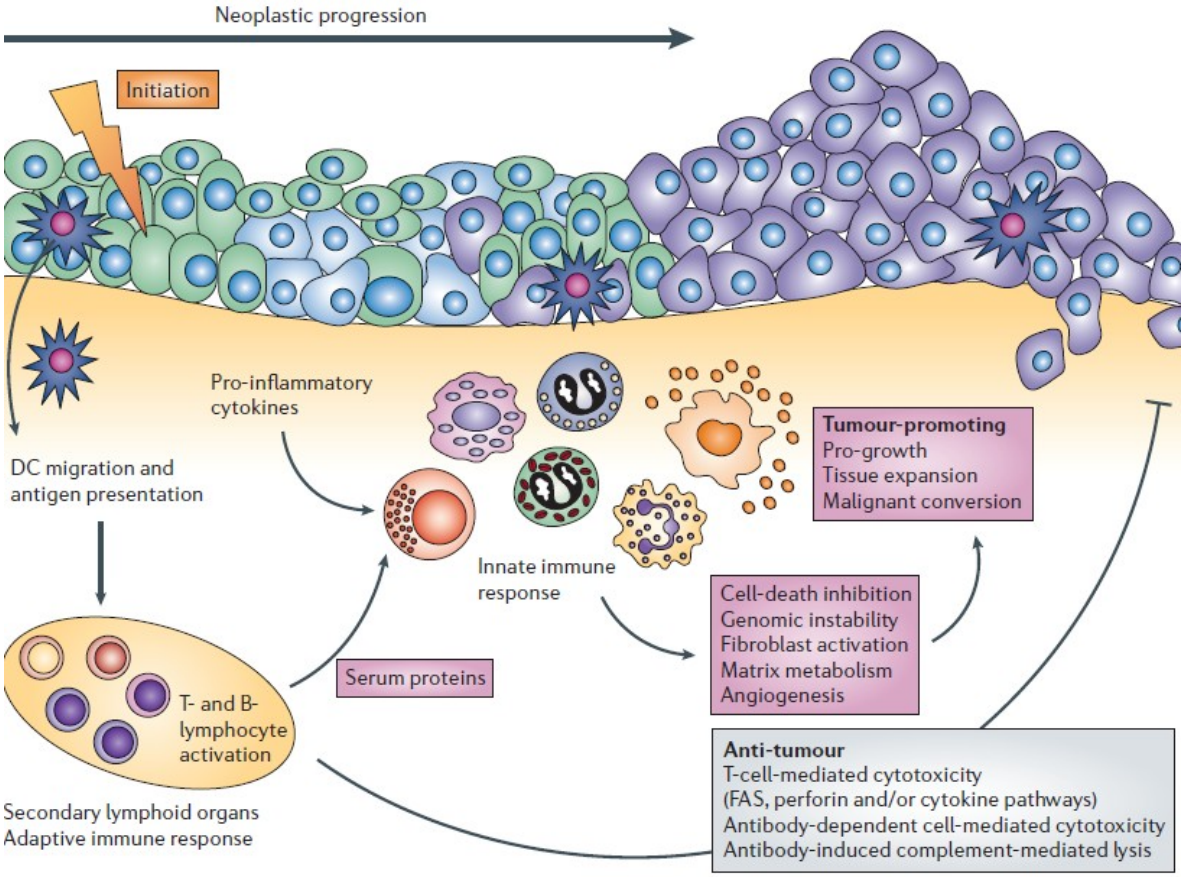

Figure 5. A model of immune-cell function during inflammation-associated cancer development[41]: *Antigens presented by cells in early neoplasmic tissues are transported to lymphoid organs by dendritic cells (DCs). An activation of B cells and humoral immune responses occurs, what can lead to the chronic activation of innate immune cells in neoplasmic tissues. So activated mast cells, granulocytes and macrophages promote tumor development by release of pro-survival molecules. In addition, inflammatory cells positively influence tissue remodeling and angiogenesis by production of pro-angiogenic mediators and extracellular proteases.*

Dramatic disruption of tissue architecture and tissue remodeling is associated with the ability of transformed cells to invade across natural barriers and to metastasize. Since this tissue disruption causes generation of proinflammatory signals in immune cells, cancers are constatly confronted with inflammatory

responses as they invade adjacent tissues.[56a] On the other hand, inflammation caused by non-cancer processes can promote the transformation of healthy tissue to malignancies.[41] An inflammation-associated cancer development is discussed in Figure 5 (page 21).

1.4.1. Tumor immunotherapy

Since combating cancer encounters several difficulties originating from the heterogenity of tumor cells, their resemblance to unaltered tissue and genetic unstability, complex approach such as a varying combination of chemotherapy, radiotherapy, immunotherapy and surgery technics may be needed to develop an effective cancer therapy. Although tumor immunotherapy is a promising modality to prevent cancer growth and reccurence, it is facing numerous challenges such as the need to identify potent and broadly expressed antigenic targets, and modulate the immune response in terms of selective identification and elimination of altered cells.[42] Cancer immunotherapies can be divided to active immunization and to passive transfer approaches. *In vitro* generation of anti-tumor lymphocytes derived from single starting cells selected for their high avidity for tumor antigens and their transfer to patient belongs to the most conventional among passive approaches. The genetic modification of these lymphocytes to improve their anti-tumor efficacy is another option for enhancement of immune responses towards tumor cells.[43] Cancer vaccination is based on immunization with either autologous or allogenic tumor antigens, whole cancer cells or cancer cell extracts.[42]

Another approach to tumor immunotherapy is based on modulation of immune system responses by different cytokines mediating *in vivo* regulation of immune responses. Administration of IL-2 to patients with metastatic kidney cancer or melanoma serves as an example of such immunotherapies.[44] Besides above mentioned approach, immune responses can be regulated by ligands of the receptors of immune cells that upon their binding to the receptor enable modulation of immune response. Since multimeric presentation of such ligands is often necessary for cross-linking of the receptor, dendrimeric structures or conjugates presenting several identical or various ligands in their structures have been synthetized.

2. AIMS OF THE WORK

The aim of this work was to describe *in vitro* effect of several carbohydrate dimers used as ligand mimetics for the receptors of NK cells on peripheral blood mononuclear cells from immunological point of view. The evaluation of the action of these ligand mimetics on PBMC was based on:

- monitoring of cellular signalization via inositolphosphate and Ca^{2+} ion production,
- natural killing of leukemic cell lines by peripheral blood mononuclear cells activated by the tested compounds,
- observation of activation-induced apoptosis of PBMC.

3. EXPERIMENTAL

3.1 Material

3.1.1. Technical equipment

Agarose Gel Electrophoresis Apparatus	*Sigma, USA*
Bürker Counting Chamber	*Marienfeld, Germany*
Centrifuge J-6M (rotor JS-5.2)	*Beckman, USA*
Centrifuge PK110 (rotor O-G26)	*ALC, USA*
Centrifuge Z 233 MK-2 (rotor 220.87 VO5/6)	*Hermle, Germany*
CO_2 incubator IGO 150 Cell Life	*Jouan, France*
Digital Analytic Scales Toledo-AL54-IC	*Mettler, Switzerland*
Fluorescent Cytometer LSR II	*BD Biosciences, USA*
Heating module Thermoblock Reacti-Therm	*Pierce, USA*
Laminar Air Flow Cabinet MSC 12	*Jouan, France*
Liquid Scintillation and Luminiscence Counter Trilux 1450 Microbeta	*Wallac, Finland*
Microplate reader Safire^2	*Tecan, Austria*
pH-meter Φ 200	*Beckman, USA*
Scale HF-1200G	*A&D, USA*
UV-transilluminator G:Box	*Syngene, UK*
Vacuum Concentrator RC 10.10	*Jouan, France*
Vortex Zx^3	*Velp Scientifica, Italy*

3.1.2. Chemicals

100bp DNA ladder N3231S	*New England Biolabs, USA*
[^{3}H]inositol (1.48 TBq/mol)	*GE Healthcare, UK*
Agarose, elfo grade	*InVitrogen, USA*
Annexin V-FITC	*Apronex Biotechnologies, CZ*
Digitonin	*Sigma, USA*
Dowex 1-X8, 100-200 mesh	*Sigma, USA*
Ethanol	*Lach-ner, CZ*
Ficoll-Paque PLUS	*GE Healthcare, UK*
Fluorescent stain Indo-1 AM	*Molecular Probes, USA*

Heparin, 5000 m.j./ml	*Léčiva, CZ*
Hoechst 33258	*InVitrogen, USA*
Ionomycin	*Sigma, USA*
Liquid Scintillant BCS	*GE Healthcare, UK*
PCR Ethidium Bromide	*Top-Bio, CZ*
Phenol	*Lach-ner, CZ*
RNAse A	*Sigma, USA*
Sodium acetate	*Lach-ner, CZ*
Trichloroacetic acid (30%)	*Sigma, USA*
Triton X-100	*Serva, Germany*

All of the chemicals were used in the highest purity grade available, mostly p.a. or research grade. Phenol and RNAse A were prepared in accordance with the standard protocols[45]. Sodium acetate was used in the form of 3.0M solution pH 5.2.

3.1.2.1. Tested carbohydrate dimers

The synthesis of all carbohydrate dimers is described in paper enclosed in Appendix 4S. These compounds were numbered consequtively in the order of their synthesis, and only biologically active (deprotected) compounds were tested in the immunological assays.

- Chemical formula of compound **3**, **7** and **8** is shown on figure 6.

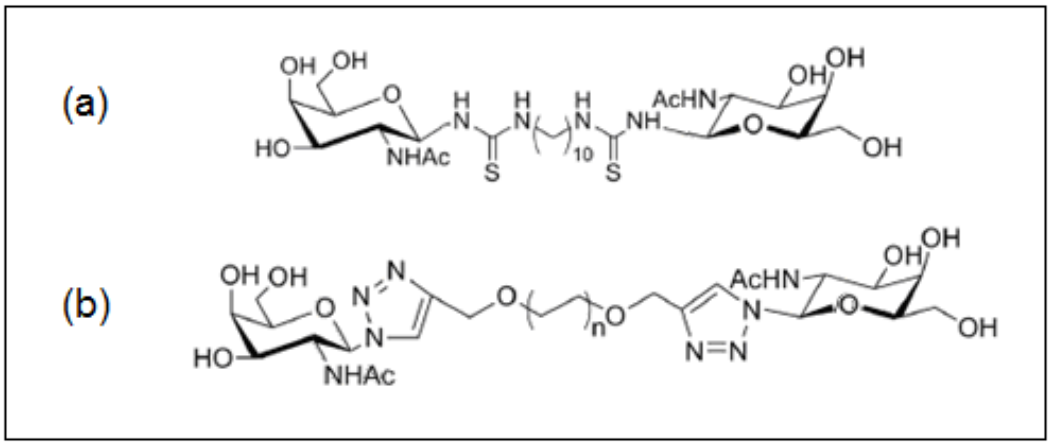

Figure 6. Chemical structure of compound 3, 7 and 8: (a) *Chemical formula of compound **3** is shown.* ***(b)*** *Chemical formula of compound **7** is constructed if **n** = **1**. Chemical structure of compound **8** is constructed by the substition of **n** for **2**.*

- Chemical structure of compounds **11**, **12** and **14** is shown on figure 7.

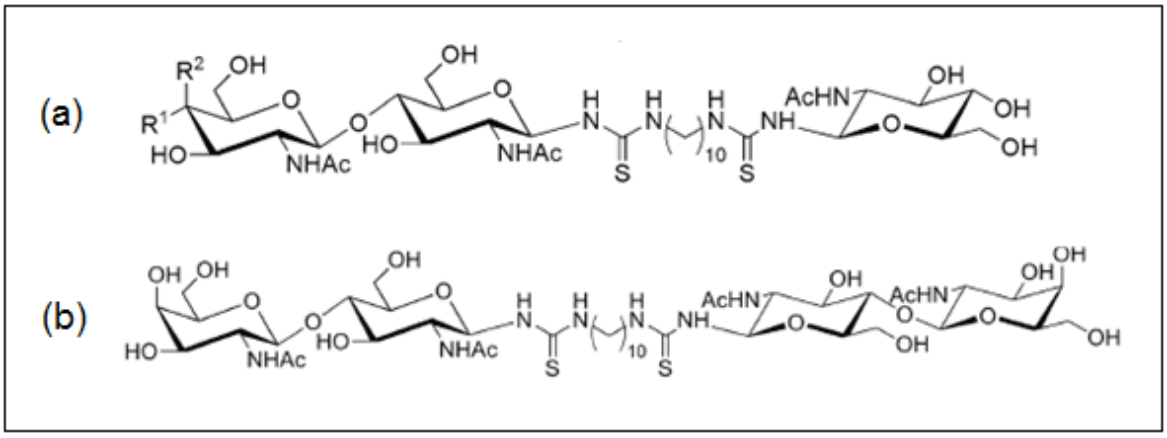

Figure 7. Chemical structure of compounds 11, 12 and 14: (a) *Compound **11** is constructed by substitution of **R^1** for **OH** group and **R^2** for hydrogen. In the case of compound **12**, **R^1** = **H** and **R^2** = **OH**.* ***(b)*** *Chemical formula of compound **14**.*

- Chemical formula of compound **16** and **17** is described on figure 8.

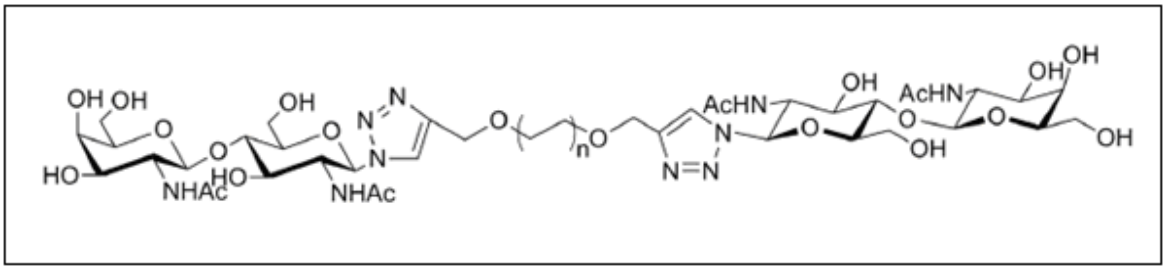

Figure 8. Chemical structure of compounds 16 and 17: *Chemical formula of compound **16** is constructed when **n** = **1** (**n** = **2** for compound **17**, respectively).*

3.1.3. Media and buffers

- DMEM medium: prepared in *ÚMG AV ČR* in accordance with the standard protocols.
- HEPES/NaCl buffer pH 7.4: 10mM HEPES, 0.15M NaCl, pH adjusted to 7.4 using HCl
- Loading buffer for agarose electrophoresis type IV: prepared in accordance with the standard protocol[45].
- Lytic buffer: 20mM Tris, 10mM EDTA, 1% Triton X-100, pH 8.0
- PBS buffer pH 7.4: 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.76mM KH_2PO_4, pH adjusted to 7.4 using HCl or NaOH

- RPMI-1640: 100 ml of 5x RPMI-1640, 10 ml of 200mM l-glutamine, 10 ml of 7.5% $NaHCO_3$, 5 ml of penicillin in activity 10000 U/ml and streptamycin in concentration 10000 μg/ml, 5 ml of 25% glucose, 5 ml of 1.1% sodium pyruvate, 5 ml of 1M HEPES pH 7.2, 25 ml of FBS – diluted with sterile water up to the volume of 500 ml. 5x RPMI-1640 concentrate and solutions of antibiotics were obtained from ÚMG AV ČR.
- TAE buffer: prepared in accordance with the standard protocol[/45/] (40mM Tris-acetate, 1mM EDTA)
- TE buffer pH 8.0: 5mM Tris, 0.5mM EDTA, pH adjusted to 8.0 using NaOH
- Türk solution: a little amount of Crystal Violet dissolved in 45 ml of 1% acetic acid

3.1.4. Biological material

- Standard blood fraction enriched in leukocytes from healthy donors (obtained from the Blood Transfusion Unit in Thomayer University Hospital)
- ^{51}Cr-labeled leukemic cells of K562 cell line in complete RPMI medium

3.2. General methods

3.2.1. Isolation of mononuclear cells from peripheral blood

Standard blood fraction enriched in leukocytes (buffy coat) was poured into a sterile 250 ml bottle and diluted with RPMI-1640 up to the volume of 140 ml. 140 μl of heparin was added immediately after the dilution and the solution was stirred. 35 ml of diluted buffy coat was gently overlayed over 15 ml of Ficoll-Paque PLUS in 4 50-ml Falcon tubes. Tubes were then spinned in Beckman J-6M centrifuge using JS-5.2 rotor at 1540 rpm at temperature 22 °C with acceleration 5 and decceleration 7 for 22 minutes. After the centrifugation, several phases were apparent in the tubes – upper phase consisting of plasma and thrombocytes and lower phase containing Ficoll and granulocytes were separated by a ring of PBMC. A pellet consisted mainly of erythrocytes. The upper phase was carefully sucked away and discarded. The ring of PBMC at the interface of all four tubes was carefully collected with pipette into a falcon tube and diluted with DMEM up to the volume of 50 ml. The tube was then spinned in ALC PK-110 centrifuge using ALC O-G26 rotor at 3000 rpm for 10

minutes to remove Ficoll-Paque. Supernatant was discarded and the pellet was resuspended in few millilitres of DMEM. The suspension of PBMC was then diluted with DMEM up to 40 ml. The tube was centrifuged again in ALC PK-110 centrifuge using ALC O-G26 rotor at 1100 rpm for 20 minutes. In this process the low speed allows PBMC to pellet while the thrombocytes stay in supernatant. The supernatant was discarded and the pellet was thoroughly resuspended in 20 ml of RPMI-1640. The average number of PBMC isolated from buffy coat was estimated in haemocytometer. 50 µl of cell suspension was mixed with 450 µl of Turk solution and the cells were counted in a Bürker chamber. 20 ml of cell suspension was transferred from falcon tube to 150 cm^2 cultivation flask and diluted with appropriate volume of RPMI needed to reach cell density 5 x 10^6 cells/ml. The cultivation flask was incubated overnight at the temperature of 37 °C and 5 % of CO_2. During this time period, adherent cells (mainly monocytes) attach to plastic surface and by these means can be eliminated from the suspension. The next day the suspension of PBMC was moved to another 150 cm^2 cultivation flask and the concentration of PBMC was estimated using Bürker chamber as described earlier.

3.2.2. Inositolphosphate production

Incorporation of [^{3}H]inositol into the cell membrane was achieved by incubation of human PBMC (10^7 cells/ml) with 100 µl of [^{3}H]inositol (1.48 TBq/mol, 37 MBq/ml) at 37 °C and 5 % of CO_2 for 3 hours. After extensive washing with DMEM the PBMC were resuspended in RPMI to reach the concentration 10^8 cells/ml. 50 µl of this suspension containing 5 x 10^6 cells/ml was placed into a well of 96-well microtiter plate heated at 37 °C and mixed with 50 µl of the solution of the tested compounds of concetration 2 µmol/l. The cells were then incubated with tested compounds for 0; 2; 4; 6; 8 and 10 minutes. The reaction was stopped at indicated times by a rapid tranfer of the reaction mixture into a well containing 100 µl of 20% trichloroacetic acid and the 96-well microtiter plate was left to incubate at 37 °C overnight. The next day the reaction mixture was neutralized by the addition of 30 µl of triethylamine. 20 µl of 50% slurry of Dowex 1-X8 in formate form was then added. Supernatant was discarded and inositol bisphosphates (inositol trisphosphates) were eluted by the addition of 50 µl of 0.3M (0.6M) ammonium formate pH 7.0. The eluent was dried in a thin-walled 96-well microtiter plate and 100 µl of Biodegradable

Counting Scintillant was added into the wells. The radioactivity was counted using the Microbeta counter.

3.2.3. Intracellular Calcium Monitoring

The cell suspension containing 10^7 PBMC was incubated with 5 pM Indo-1 AM in complete RPMI-1640 for 1 hour. Cells were washed twice with HEPES/NaCl buffer pH 7.4 and resuspended in fresh RPMI medium to concentration 5 x 10^6 cells/ml. 50 µl of the cell suspension loaded with calcium-sensitive fluorescent dye Indo-1 was pipetted into the wells of 96-well microtiter plate tempered at 37 °C. 50 µl of HEPES/NaCl buffer was added into the first two wells and their content was transferred to the microtiter plate wells containing 20 µl of 1% digitonin in PBS buffer. 50 µl of 2µM solution of the tested compounds was added into the rest of the wells and the cell suspension with tested compounds in individual wells was then transferred to 20 µl of 1% digitonin in PBS buffer in one minute intervals (total of 10 minutes). 10 µl of 10µM ionomycin was added into last two wells. The 96-well microtiter plate was then spinned in Beckmann J-6M centrifuge with JS-5.2 rotor at 2600 rpm at temperature 4 °C with acceleration 9 and decceleration 9 for 5 minutes. Supernatant from the wells was then transferred to a 96-well plate UV-Star and was left to incubate in the dark. The next day, the fluorescence was measured on Safire^2 spectrofluorimeter using an excitation wavelenght of 349 nm and emission wavelength 430 nm. Concentration of intracellular Ca^{2+} ions was calculated using the formula:

$$C_{Ca^{2+}}\,[nM] = 250 \times \frac{F - F_{min}}{F_{max} - F}$$

where F is the measured fluorescence, *250 (nM)* is the dissociation constant of Indo-1, F_{min} refers to the minimal fluorescence after the addition of 10 mM EGTA and sufficient Tris base to raise pH to >8.3 and F_{max} is the maximal fluorescence after the lysis of the Indo-1-loaded cells with Triton X-100 (0.07%).

3.2.4. Natural Killing

10^4 ^{51}Cr-labeled leukemic cells of K562 cell line (target cells) in 100 µl of complete RPMI were pipetted into the wells of round-bottomed 96-well plate. 50 µl of 5µM solution of the tested compounds was added to these target cells in triplicates. Thereafter, the appropriate amount of PBMC (effector cells) was added in 100 µl suspension to reach the requested ratio of effector to target cells E:T 30:1, 100:1, 300:1 and 1000:1.

	1	2	3	4	5	6
A	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI
B	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI
C	Compound 3 E:T = 30:1	Compound 3 E:T = 30:1	Compound 3 E:T = 30:1	Compound 3 E:T = 100:1	Compound 3 E:T = 100:1	Compound 3 E:T = 100:1
D	Comp. 11 E:T = 30:1	Comp. 11 E:T = 30:1	Comp. 11 E:T = 30:1	Comp. 11 E:T = 100:1	Comp. 11 E:T = 100:1	Comp. 11 E:T = 100:1
E	Comp. 12 E:T = 30:1	Comp. 12 E:T = 30:1	Comp. 12 E:T = 30:1	Comp. 12 E:T = 100:1	Comp. 12 E:T = 100:1	Comp. 12 E:T = 100:1
F	Comp. 14 E:T = 30:1	Comp. 14 E:T = 30:1	Comp. 14 E:T = 30:1	Comp. 14 E:T = 100:1	Comp. 14 E:T = 100:1	Comp. 14 E:T = 100:1
G	Compound 7 E:T = 30:1	Compound 7 E:T = 30:1	Compound 7 E:T = 30:1	Compound 7 E:T = 100:1	Compound 7 E:T = 100:1	Compound 7 E:T = 100:1
H	Compound 8 E:T = 30:1	Compound 8 E:T = 30:1	Compound 8 E:T = 30:1	Compound 8 E:T = 100:1	Compound 8 E:T = 100:1	Compound 8 E:T = 100:1

	7	8	9	10	11	12
A	Comp. 16 E:T = 30:1	Comp. 16 E:T = 30:1	Comp. 16 E:T = 30:1	Comp. 16 E:T = 100:1	Comp. 16 E:T = 100:1	Comp. 16 E:T = 100:1
B	Comp. 17 E:T = 30:1	Comp. 17 E:T = 30:1	Comp. 17 E:T = 30:1	Comp. 17 E:T = 100:1	Comp. 17 E:T = 100:1	Comp. 17 E:T = 100:1
C	E:T = 30:1	E:T = 30:1	E:T = 30:1	E:T = 100:1	E:T = 100:1	E:T = 100:1
D	E:T = 30:1	E:T = 30:1	E:T = 30:1	E:T = 100:1	E:T = 100:1	E:T = 100:1
E	E:T = 30:1	E:T = 30:1	E:T = 30:1	E:T = 100:1	E:T = 100:1	E:T = 100:1
F	E:T = 30:1	E:T = 30:1	E:T = 30:1	E:T = 100:1	E:T = 100:1	E:T = 100:1
G	E:T = 30:1	E:T = 30:1	E:T = 30:1	E:T = 100:1	E:T = 100:1	E:T = 100:1
H	Triton X-100	Triton X-100	Triton X-100	Triton X-100	Triton X-100	Triton X-100

Figure 9. Arrangement of microtiter plate for natural killing assays: *10^4 chromium labeled leukemic cells in 100 µl was pipetted into the wells. 50 µl of PBMC suspense of a given concentration was added into the*

wells. Where indicated, 50 µl of 5µM solution of tested compounds was added. Triton X-100 was added to indicated wells after 3 hours of incubation. Analogous microtiter plate was designed for E:T ratio 300:1 and 1000:1.

Arrangement of the 96-well microtiter plate can be seen on Figure 9 (page 30). Where indicated, 50 µl of RPMI was added instead of the tested compounds. The plate was then placed into a tissue incubator and incubated at 37 °C and 5 % of CO_2 for 3 hours. 50 µl of 1% Triton X-100 was then added into the maxima release wells and the incubation continued for 1 hour. After the incubation, 50 µl of supernatant was collected and transferred to another 96-well microtiter plate and its content was left to dry. The next day 100 µl of Biodegradable Counting Scintillant was added into the wells. The radioactivity was counted using the Microbeta counter. Specific cytotoxicity was calculated from obtained data using the formula:

$$Spec.Cyt.\ [\%] = 100 \times \frac{EXP - SPONT}{MAX - SPONT}$$

where *EXP* are the counts in experimental wells, *SPONT* counts in wells containing medium instead of effector cells and *MAX* refers to the counts in the wells containing 1% Triton X-100. Killing curves were then constructed, from which the L.U. (lytic unit) counts were calculated. Thereafter, the lytic efficiency defined as the inverse of the lytic unit count was estimated.

3.2.5. Apoptosis assays – flow cytometry

90 µl of suspension of PBMC in RPMI of concentration 1,11 x 10^6 cells/ml was pipetted into the round-bottomed 96-well plate. The cells were incubated with 10 µl of 10µM solution of the tested compounds at 37 °C and 5 % of CO_2 for 12 (eventually 6) hours. The percentage of apoptotic cells was estimated using Annexin V-FITC/Hoechst 33258 staining and flow cytometry. The percentage of early (Annexin V-FITC$^+$/Hoechst 33258$^-$) and late (Annexin V-FITC$^+$/Hoechst 33258$^+$) apoptotic cells observed in the presence of PBS or in the presence of 5 x 10^{-6}M arsenite was used as negative and positive control.

3.2.6. Apoptosis assays - DNA ladder

900 µl of suspence of PBMC in RPMI of concentration 5,56 x 10^6 cells/ml was pipetted into 24-well plate. The cells were incubated with 100 µl of 10µM solution of the tested compounds at 37 °C and 5 % of CO_2 for 12 hours. After the incubation, the suspension of PBMC with the tested compounds was tranferred to Eppendorf microtubes and spinned in Hermle Z233 MK-2 centrifuge with 220.87 VO5/6 rotor at 2000 rpm at 4 °C for 10 minutes. Supernatant was discarded and the pellet was resuspended in 450 µl of lysis buffer. The cell suspensions were then incubated on ice for 10 minutes. Microtubes were spinned again in Hermle centrifuge at 10000 rpm for 10 minutes. 400 µl of supernatant was collected into new microtubes and 200 µl of phenol and 200 µl of chloroform was added. Microtubes were then shaked 1 hour at 4 °C and thereafter spinned in Hermle centrifuge at 12000 rpm for 10 minutes. 350 µl of upper phase was collected and transferred into new microtubes, and 175 µl of 3.0M sodium acetate of pH 5.2 and 1000 µl of 96% ethanol was added. After incubation on ice for 1 hour, the microtubes were spinned in Hermle centrifuge at 14000 rpm for 10 minutes. Supernatant was discarded and the pellet was dried in a vacuum dryer.

The pellet was then resuspended in 20 µl of RNAse (1 mg/ml) in TE buffer and incubated at 37 °C for 30 minutes. 4 µl of loading buffer for agarose electrophoresis type IV was added into the solution and the samples were loaded onto 2% agarose gel containing trace of ethidium bromide (1 g of agarose per 50 ml of TAE buffer). Electrophoresis was run under the constant voltage of 100 V in TAE buffer. The gel was photographed in transilluminator G:Box using an excitation wavelenght of 302 nm and emission wavelength 595 nm.

4. RESULTS

4.1. Description of the tested compounds

The compounds tested in the presented work were developed as a part of a long time project aimed at finding suitable ligand mimetics for activating receptors of natural killer cells. The first generation of these mimetics was represented by liposomes coated with lipid-linked carbohydrate sequences. The second generation was represented by dendrimers in which the highly branched structures were coated with carbohydrates. The current generation of these ligand mimetics is based on carbohydrate dimers in which mono- or disacharides are attached to a linker of defined length using either thiourea or triazole chemistry giving rise to the respective (thiourea or triazole) series.

The thiourea series is based on compound **3** in which the length of the linker was optimized previously. The optimal length for activation of natural killer cells was found to be decyl -$(CH_2)_{10}$-.[/46/] Additional compounds in this series were obtained by extension of the carbohydrate sequence either on one end by the addition of either β1→4 linked GlcNAc (compound **11**) or β1→4 linked GalNAc (compound **12**), or at both ends by the addition of β1→4 linked GalNAc (compound **14**). The addition of GalNAc into the terminal position is preferred to the addition of GlcNAc because of higher affinity of this carbohydrate for activating NK cell receptors.

The triazole series is based on compounds **7** and **8** that were designed and synthesized in collaboration with the University of Aarhus in Denmark (synthesis and other experiments with the tested compounds are described in article attached as Appendix 2). The presence of triazole linker instead of thiourea linker represents an advantage from the point of view of increased affinity for the receptors. The length of the linker was not optimized for this series, nevertheless the distance of the terminal GlcNAc residues in compound **8** may be considered to be about the same as in compound **3**. Additional compounds in this series contain compounds **16** and **17** that are derived from compounds **7** and **8** by extending them with β1→4 linked GalNAc on both ends. In addition to the above new compounds tested in the present work, the standard dimer of N-acetyllactosamine tested previously[/47/] has been included as positive control.

4.2 Effect of the tested compounds on the precipitation of soluble NK cell receptors, mouse NKR-P1A and human CD69

Since the standard inhibition and binding assays that are routinely used for the evaluation of biological activity of the monovalent ligands may not provide reliable results for the multivalent compounds, we have decided to apply an alternative assay that measures the amount of soluble recombinant receptor precipitated by individual compounds. In this experiment, soluble dimeric carbohydrate ligands are mixed with soluble dimeric recombinantly produced NK cell receptors. In case of efficient binding of individual compounds to binding sites within the receptor molecules, a standard precipitation reaction occurs resulting in the formation of receptor oligomers that are much less soluble compared to the unbound receptor molecules. The conditions of the precipitation assays are fine tuned optimizing the concentration of the precipitant (polyethyleneglycol) to achieve maximum precipitation by the positive control compound, and minimum precipitation by the negative control (usually PBS).

Using the above optimized assays, the thiourea dimerized carbohydrates **3**, **11**, **12**, and **14** had very little effect on the precipitation of soluble rat NKR-P1 (Figure 10 at page 35, *top panel*). This is in accordance with previous results obtained in the laboratory indicating that dimeric thiourea linked decyl GlcNAc had very little precipitating activity towards the rat NKR-P1A[/48/]. Extending this particular carbohydrate dimer with another β1→4 linked carbohydrate residue on one or both ends did not have any particular effect in this case. On the other hand, results obtained using the triazol linker were more encouraging: maximum precipitation activity was achieved by compound **7** followed by compound **8**, and **17**. Compound **16** was completely negative in the precipitation assays under the used experimental conditions (Figure 10, *top panel*).

Results obtained with human CD69 in the precipitation assays were completely different. Although the dimeric GlcNAc (compound **3**) did not provide much of the precipitation either, there has been some precipitation with the extended variants (compounds **11** and **12**), and very high level of precipitation was achieved with the disaccharides containing compound **14**. Similarly, the triazole compounds provided significant levels of precipitation, although the hierarchy appeared to be reversed when compared with rat NKR-P1: maximum level of precipitation was

obtained using the extended carbohydrate dimers with terminal disaccharide groups (Figure 10, *bottom panel*).

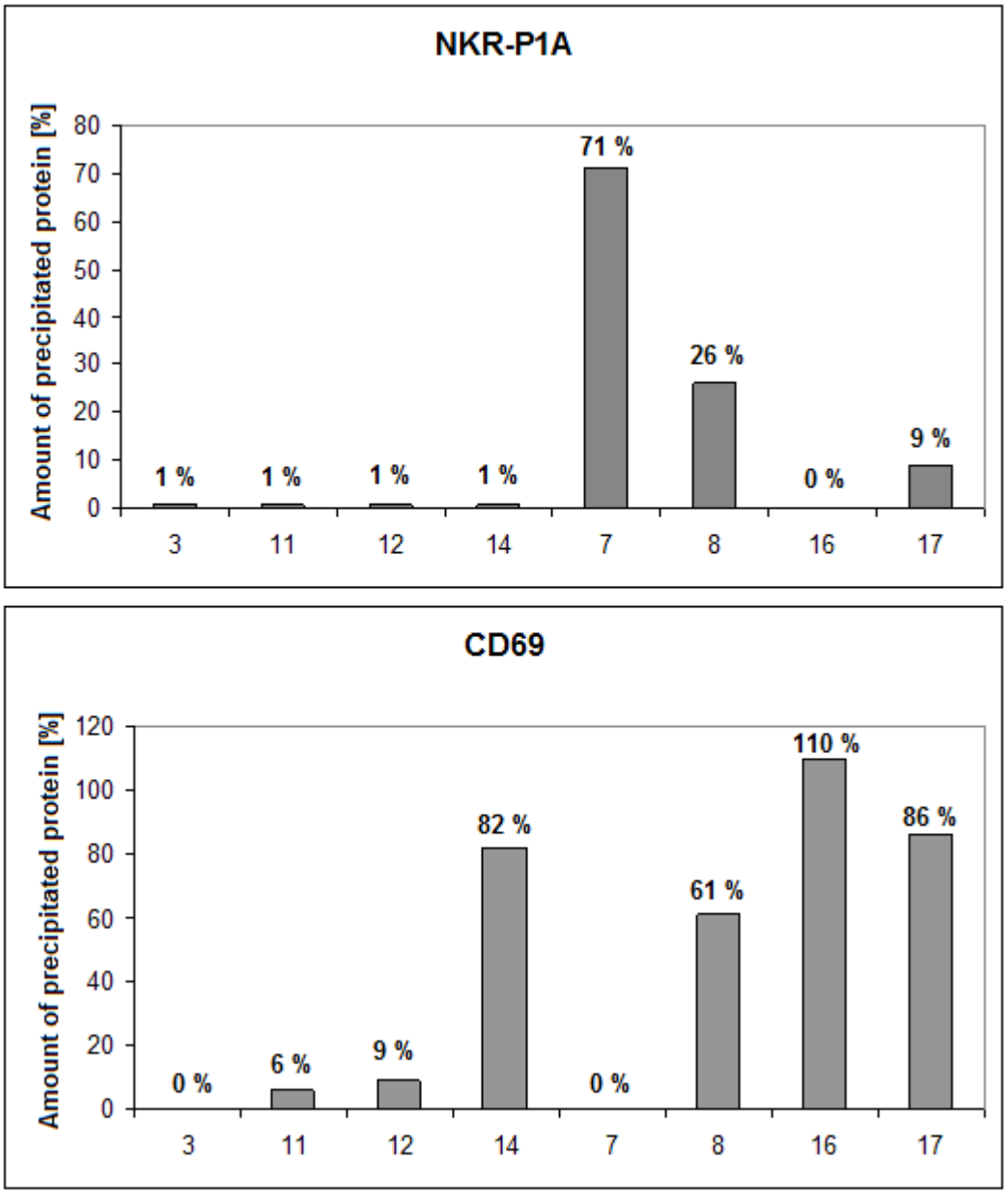

Figure 10. Precipitation assays of soluble NKR-P1A and CD69 receptors: *Precipitation assays follow the ability of the tested compounds to precipitate the respective recombinant NK cell receptor proteins from solution. Top panel shows the ability of the tested compounds to precipitate recombinant soluble NKR-P1A and bottom panel shown the precipitation ability of the tested compounds on human soluble CD69.*

4.3. Cellular activation assays

The next stage of testing of biological effects of carbohydrate dimers was performed using isolated immune cells expressing the cellular forms of NK cell receptors at their surface. Since rat NK cells freshly prepared from rat spleens were not available and with regard to the potential use of the tested compounds in human cancer immunotherapies, we have decided to test their effect on human $CD69^+$ lymphocytes. These cells were isolated from leukocyte-enriched fractions obtained from blood donors at the local Blood Transfusion Unit. Leukocyte fraction was diluted by medium, and the fraction of peripheral blood mononuclear cells composed mostly of B and T-lymphocytes was obtained by Ficoll-Paque density gradient centrifugation.

An effective means of quantization of cellular signaling is the measurement of production of inositolbisphosphates, inositoltrisphosphates and concentration of intracellular calcium ions. Therefore the first assays performed were standard lymphocyte activation assays based on the production of inositolphosphates and elevation of intracellular calcium. **PBS** was used as a negative control and the dimeric *N*-acetyllactosamine disaccharide **diL** was used as a positive control.

4.3.1. Inositolphosphate production

As it was mentioned in section 1.2.2., phosphatidylinositol-4,5-bisphosphates present in the cytoplasmic membrane of cytotoxic lymphocytes are upon cellular activation hydrolyzed by phospholipase C to DAG and inositol-1,4,5-trisphosphate. These molecules then act as second messengers and evoke several cellular responses such as calcium influx, what then regulates a range of processes such as cell survival, proliferation, cytoskeletal rearrangement and vesicle trafficking. Inositol-1,4,5-trisphosphate is then dephosphorylated by a specific 5-phosphatase to inositol-1,4-bisphosphate.

In this experiment, incorporation of inositol containing radiolabeled ^3H isotope to cytoplasmic membrane of effector cells in the form of PIP_2 was achieved by incubation of PBMC with [^{3}H]inositol according to the method described in section 3.2.2.. The amount of [^{3}H]inositol released into the cytoplasm of an effector cell upon activation caused by the tested compounds in specific time periods was evaluated by measurement on Liquid Scintillation counter Microbeta. Graphic dependance of the

concentration of Ins2P and Ins3P on time for individual compounds was created and can be seen in Appendix 1.1. When looking at these time curves, it is evident that the maximum concentration of Ins3P was achieved between 2 and 4 minutes, while in the case of Ins2P, the maximum concentration was achieved later – between 4 and 6 minutes. From these complete time course experiments, maximal values between 4 and 6 (Ins2P) and between 2 and 4 (Ins3P) minutes for individual compounds were extracted. These data are illustrated in Figure 11.

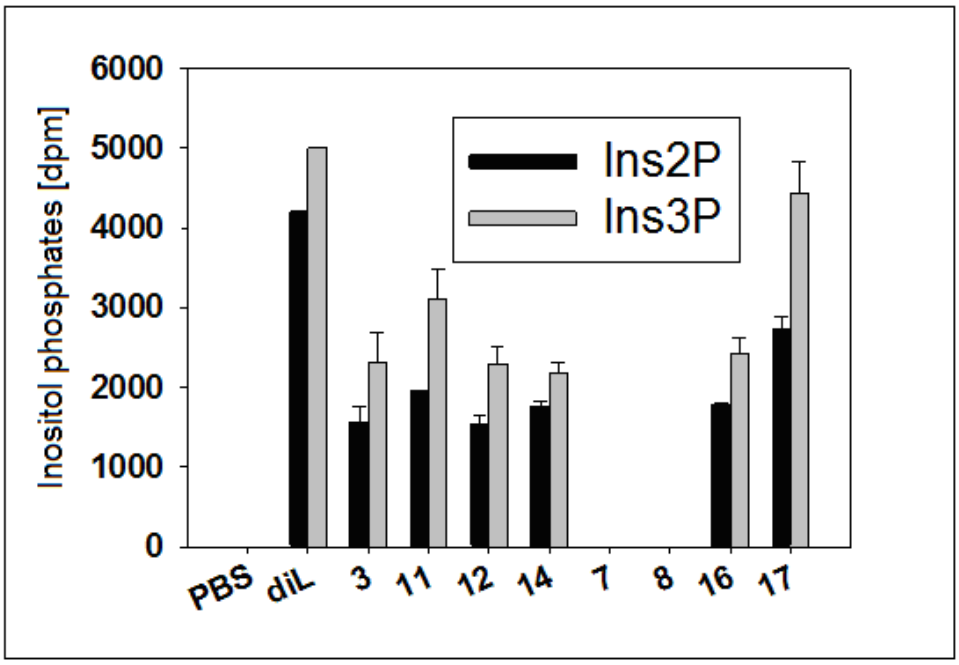

Figure 11. The inositolphosphate production by CD69^{+} human lymphocytes in the presence of 10^{-10}M concentrations of the tested compounds: *Individual compounds were mixed with $5x10^{6}$ lymphocytes at the indicated final concentrations in a total volume of 100 µl. The reaction was heated to 37 °C, and the production of inositolbisphosphates (Ins2P) and inositoltrisphosphates (Ins3P) was determined after 1 min, 2 min, 4 min, 6 min, 8 min and 10 min of heating as described in the experimental section. From the complete production curves, only the peak values between 2-4 (Ins3P) and 4-6 (Ins2P) minutes were extracted for individual compounds. Average values +/- ranging from duplicate experiments are shown.*

When considering compounds of thiourea series, all of the tested compounds caused an increase in phosphoinositol production. The most significant increase was observed upon activation with compound **11**. Compounds **7** and **8** belonging to triazole series showed no effect at all on phosphoinositide signaling. On the other hand, compound **17** proved its qualities as a molecule with a high potential to activate human PBMC as it scored the best in inositolphosphate production assays.

4.3.2. Intracellular calcium monitoring

After evaluation of data concerning the inositolphosphate production, an experiment monitoring intracellular Ca^{2+} concentration was performed. Since Ca^{2+} ions act as a second messenger in phosphoinositide signaling, its intracellular concentration can be linked to an extent to which an effector cell is activated. At first, PBMC were loaded with flourescent dye Indo-1 AM that is widely used for measurement of the concentration of Ca^{2+} in biological systems. Indo-1 AM combine a stilbene fluorophore with the octacoordinate, tetracarboxylate pattern of liganding groups characteristic for EGTA, that allows an increase in selectivity against magnesium and heavy metals, along with the stronger flourescence and wavelength shifts upon Ca^{2+} binding.[49] Indo-1 AM is a membrane-permeant ester derivative whose ester groups are splitted by cytosolic esterases what then leaves the membrane-impermeant Indo-1 trapped in the cytosol.[50]

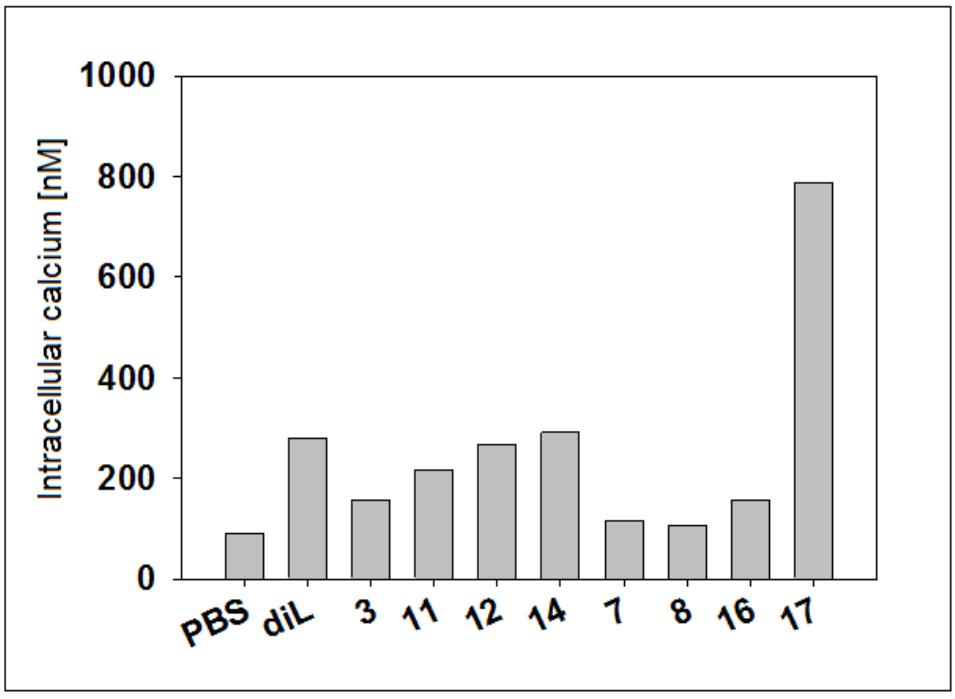

Figure 12. Evaluation of the effects of the tested compounds on the increase of intracellular calcium in CD69$^+$ human lymphocytes: *Indicated compounds were mixed with 5x10^6 lymphocytes, and the levels of intracellular calcium were monitored for 10 min in 1 min intervals. Maximal values observed 3rd minute after the addition of individual tested compounds are shown. The values are averages from three independent experiments with S.D. between measurements not exceeding 5 % of the measured values. The addition of PBS was used as a negative control. The concentration of intracellular calcium in this case is approximately 100 nM, what is in correspondence with values stated in literature.*

Indo-1 loaded peripheral blood mononuclear cells were equilibrated at 37 °C for 2 or 3 minutes, and 2 samples were withdrawn for the determination of intracellular calcium levels under basal conditions. Thereafter, the tested compounds were mixed fast with equilibrated cells in the 10 remaining wells, and samples were withdrawn in 1 min intervals. Intracellular calcium levels were determined as described in the section 3.2.3. After 10 minutes of measurements, ionomycin (compound opening the calcium channels in plasma membrane, 10 μM) was added into the last two wells, and maximum increase of calcium was measured to verify the experimental system. Concentration of Ca^{2+} ions released in PBMC was counted using an equation introduced in section 3.2.3. and these data were used to create a plot showing a dependance of Ca^{2+} concentration on time for individual compounds. These figures can be seen in Appendix 1.2. Maximal values of Ca^{2+} concentration were observed 3 minutes after the addition of the tested compounds and these data were used to create figure 12 (page 38) that illustrates the maximal concentration of Ca^{2+} ions released in peripheral blood mononuclear cells upon incubation with the tested compounds.

The obtained results are in fact somewhat paralel to the results based on inositolphosphate production determinations, what is often observed under the used experimental conditions except that **diL** positive control did not produce such a significant increase in intracellular calcium levels. As figure 12 illustrates, the highest concentration of intracellular calcium was observed upon activation with compound **17**. On the other hand, compounds **7** and **8** belonging to the same triazole series remained completely inactive in cellular activation assays. Compounds belonging to thiourea series (compounds **3**, **11**, **12** and **14**) caused some increase in intracellular Ca^{2+} concentration, even though the values were in comparison with data observed for compound **17** less significant.

4.4. Effects of the tested compounds on natural killing

The additional *in vitro* test that proved useful for the evaluation of immunological effects of the tested compounds is their influence on natural killing. Firstly, leukemic cell line K562 was incubated in medium containing radiolabeled chromium isotope ^{51}Cr to achieve its asimilation. K562 cell lines derived from a

patient with chronic myelogenous leukemia in blast phase are commonly used as targets in cytotoxicity assays because they are very sensitive to NK cell cytolysis and do not express MHC antigens.[7] We used a method described in detail in section 3.2.4. to measure the release of radiolabeled chromium from target cells that have been exposed to effector cells activated by the tested compounds. The experiment was performed with the ratio of effector to target cell E:T 30:1, 100:1, 300:1 and 1000:1. The addition of a detergent Triton X-100 was used as a positive control.

Specific cytotoxicity was counted using a formula introduced in part 3.2.4. and killing curves for individual compounds were constructed as a dependance of counted specific cytotoxicity on E:T ratio. These killing curves can be found in Appendix 1.3. Using these killing curves, L.U. counts (the number of lytic units corresponds to E:T ratio producing 50 % specific cytotoxicity, and is thus an equivalent of IC_{50} values in inhibition assays) were calculated and the lytic efficiency defined as the inverse of the lytic unit count was estimated. Relative lytic efficiency of individual compounds is shown on figure 13.

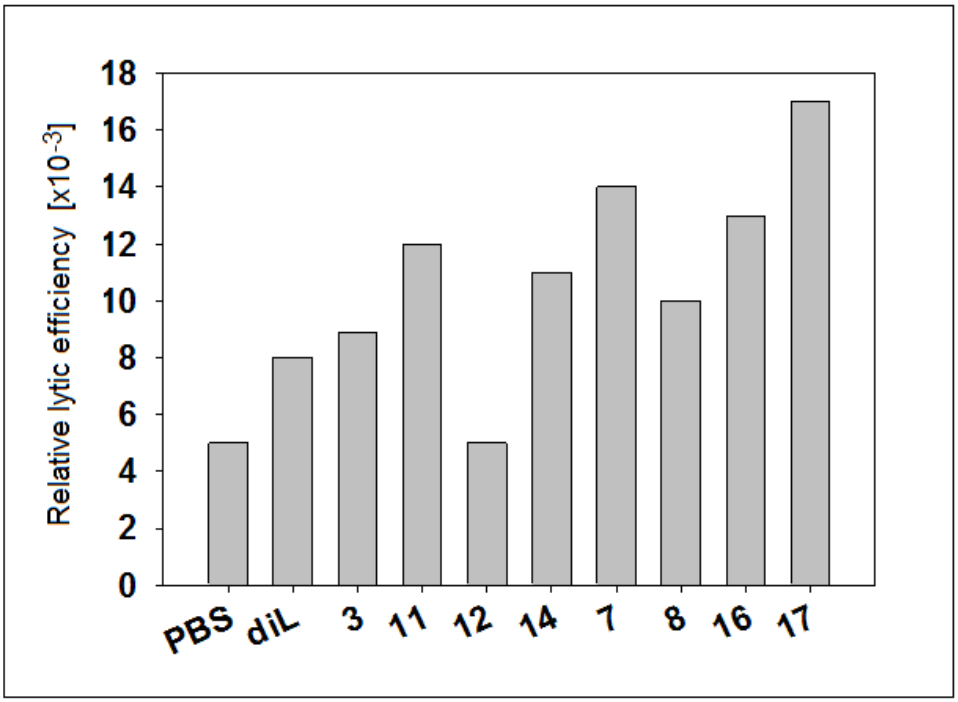

Figure 13. Effect of the tested compounds on natural killing of the sensitive human erythroleukemic cell line K562 by human CD69$^+$ lymphocytes: *The complete killing curves were recorded for E:T ratio ranging from 30 to 1000, from which the lytic units for each compound were extracted. In order to allow better comparison of the effects of individual compounds, the reciprocal values of lytic units count designated as*

Relative lytic efficiency was calculated. The values are averages from three independent determinations with the standard deviations always less than 5 % of the measured values.

In thiourea series, the most significant increase of relative lytic efficiency was achieved by activation with compounds **11** and **14**. In the case of compound **11**, the relative lytic efficiency increased twice as much as corresponding negative control. On the other hand, compound **12** showed no effect on relative lytic efficiency. The highest increase in relative lytic efficiency was achieved by activation of PBMC with compound **17** which belongs to triazole series. Since compounds **7**, **16** and **17** affected the relative lytic efficiency more than any compound in thiourea series, it can be stated that compounds of triazole series showed stronger effect on cytotoxicity of PBMC towards target cells.

4.5. Apoptosis assays

It is well known that contact of a lymphocyte with a target cell and its activation can lead to cytotoxicity-induced cell death (CICD). It is thought that secretion of lytic enzymes perforin and granzymes is calcium-dependent and therefore requires mobilization of calcium from intracellular stores. Since the reduction of intracellular calcium stores below a predetermined treshold occurs and it is known to trigger apoptosis, activation of an effector cell can lead to its death.[51] Interestingly, $CD8^+$ can escape CICD by ligation of CD8 by MHC-I molecules expressed on NK cells or eventually target cells and the subsequent CD8-induced influx of Ca^{2+} ions from extracellular space.[51] Since CICD is a significant phenomenon influencing intensity of immune response, we performed two additional experiments observing the activating or inhibitory effects of the tested compounds on apoptosis of PBMC.

4.5.1. Apoptosis evaluation using flow cytometry

In this experiment, we evaluated the effects of the tested compounds on apoptosis of human peripheral blood mononuclear cells. 10^5 PBMC in 90 µl of complete RPMI was incubated with 10µM solution of the tested compounds for 6 and 12 hours, respectively. After the incubation, cell suspensions were mixed with the fluorescent dye Annexin V that is able to bind to phosphatidylserine, and Hoescht

33258 that associates with DNA. Phosphatidylserine (PS) is a molecule that is ordinarily sequestered in the plasma membrane inner leaflet. Its appearance in the outer leaflet is a universal phenomenon in cells undergoing apoptosis. Outer leaflet PS serves as a signal for noninflammatory engulfment of apoptotic cells. The mechanism of this phenomenon is not entirely understood, even though it is proposed that it is associated with loss of aminophospholipid translocase activity and nonspecific flip-flop of various phospholipids.[52] Since early apoptosis is characterised by flip-flop of PS, early apoptotic cells will be Annexin V positive and Hoechst 33258 negative. As a cell progresses to late apoptosis, transport mechanisms and membrane permeability change what results in the leakage of Hoechst 33258 into the cell. Late apoptotic cells are therefore Annexin V and Hoechst 33258 positive.

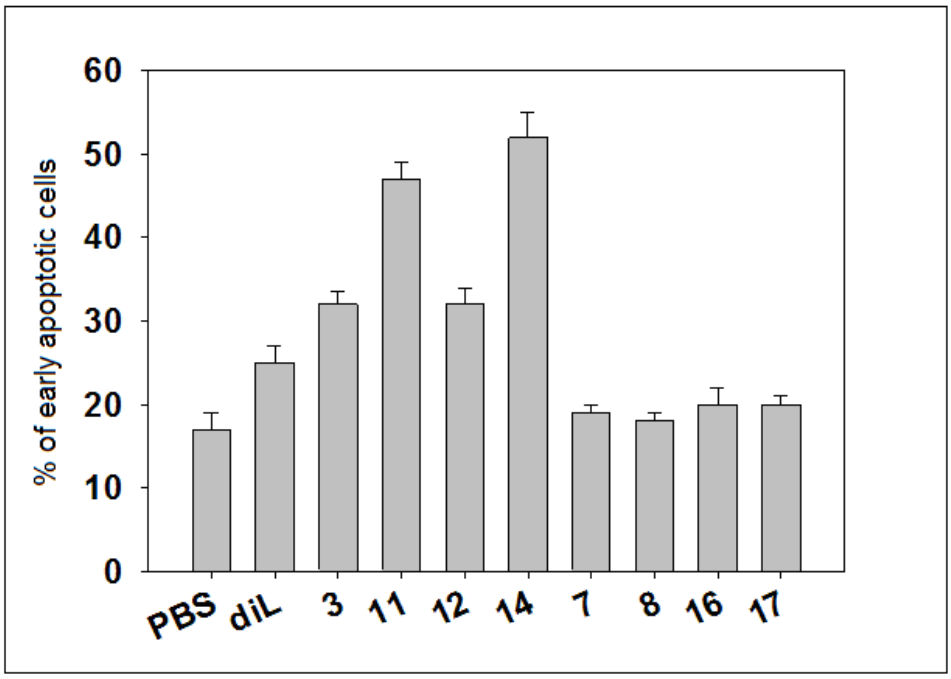

Figure 14. Activation induced apoptosis of PBMC from CD69$^+$ donors: *PBMC from donors with high expression of CD69 were incubated with the tested compounds for 12 hours. Then the percentage of early apoptotic cells (Annexin V-FITC$^+$/Hoechst 33258$^-$) was determined using flow cytometry. Results are average values +/- range from duplicate experiments.*

The percentage of early and late apoptotic cells was then determined using the flow cytometry. Complete results of this experiment are discussed in Appendix 1.4. Here, we show the ability of the tested compounds to induce activation-induced apoptosis after incubation with CD69^{high} PBMC for 12 hours (figure 14). Principal

differences were observed between the thiourea series dimers and the triazole series dimers with regard to their ability to induce apoptosis in effector cells. A gradual increase in the ability to induce activation related programmed cell death is evident in thiourea series. The highest ability to induce apoptosis in $CD69^{high}$ PBMC was observed in the case of compound **14**, followed by compound **11**. However, compounds of triazole series were devoid of this proapoptotic activity. The ratio of early apoptotic cells was approximately the same as in the case of negative control. Even highly active compounds **16** and **17** showed just a very little ability to induce activation related apoptosis.

4.5.2. Fragmentation of DNA – DNA ladder

To confirm the results described previously, an experiment by which we evaluated the DNA fragmentation originating from activation induced cell death was performed. DNA degradation to the fragments of specific length occurs in cells undergoing apoptosis. Since this DNA degradation is caspase-independent, it has been attributed to two mitochondrial proteins endonuclease G a apoptosis-inducing factor (AIF) that upon their release translocate to the nucleus and induce nuclear condensation and large scale DNA fragmentation. Nevertheless, an important role for DNAse II in DNA fragmentation was discovered, since DNAse II-deficient mice accumulated undigested DNA.[53] Fragments of DNA of specific length can be easily separated by agarose electrophoresis and upon their visualisation, DNA fragments form a structure reminding a ladder.

5 x 10^6 PBMC from $CD69^{high}$ donors in 900 µl of complete RPMI was incubated with 100 µl of 10µM solutions of the tested compounds for 12 hours. After the incubation, DNA from the cells was harvested according to the method described in detail in section 3.2.6. Isolated DNA was separated on agarose electrophoresis and DNA fragmentation was evaluated. Unfortunately, even though the experiment was performed several times, we were unable to isolate DNA fragments due to severe technical difficulties. Therefore, the results of this experiment are not included.

5. DISCUSSION

For much of the twentieth century, the possibility of tumor immunotherapy was met with scepticism and doubt. However, much has changed in the past 20 years as the knowledge of the molecular basis of tumor-host interaction increased and the new technics in biotechnology that made large scale production of biologic reagents for pharmaceutical use possible were developed. Extraordinary possibilities for the development of effective tumor immunotherapies were opened which resulted in invention of several strategies for combating cancer using the modulation of immune system responses. A new promising approach to tumor immunotherapy is based on activation of killer lymphocytes, in particular natural killer cells, through specific ligands for their surface receptors. The ligands must be sufficiently potent in order to achieve efficient activation of natural killer cells, but at the same time hyperactivation of these cells and the resulting cytotoxicity-induced cell death must be under control.

Receptor modulation based on natural (physiological) ligands and their structural analogues is the most convenient and advantageous. However, since the physiological ligands for majority of C-type lectin receptors of natural killer cells are unknown, ligand mimetics are used instead. A long time project realized in our laboratory focuses on the structure determination and characterisation of several receptors of natural killer cells, most notably CD69 and NKR-P1. Affinity of these receptors towards several different ligands on the bases of carbohydrates or peptides has been observed. Since immunomodulation therapies using these lymphocyte receptors seem to be very promising, detailed studies evaluating the effects of the ligand mimetics on peripheral blood monoclear cells are necessary.

The ligand mimetics tested in this work were based on monomeric or oligomeric carbohydrates attached to the central linker of varying length using either thiourea or triazole chemistry. As a result of previous investigations[46], the optimal linker length for cross-linking of NKR-P1 family receptors was found to be decyl attached to GlcNAc through thiourea linker (compound **3**). At the same time, efficient cross-linking of CD69 requires usually disaccharide ligands with GalNAc β1→4 GlcNAc sequence, and the length of the linker doesn't need to be optimized. Additional compounds of thiourea series were obtained by extension of the

carbohydrate sequence on one or both ends by the addition of either β1→4 linked GlcNAc or β1→4 GalNAc (for more information and chemical structure of the tested compounds see section 3.1.2.1. and 4.1.). The presence of triazole linker instead of thiourea linker in compounds in triazole series is advantageous from the point of view of increased affinity for the receptors. The importance of the linker length optimization of the ligand mimetics for interaction with NKR-P1 is described in Figure 15. In these experiments, a linker-length-dependent cooperative binding of thiourea-linked *N*-acetyl-D-glucosamine dimers to NKR-P1 responsible for activation of this receptor and enhancement of immune response towards cancer cells in mouse melanoma models was observed.[/46/]

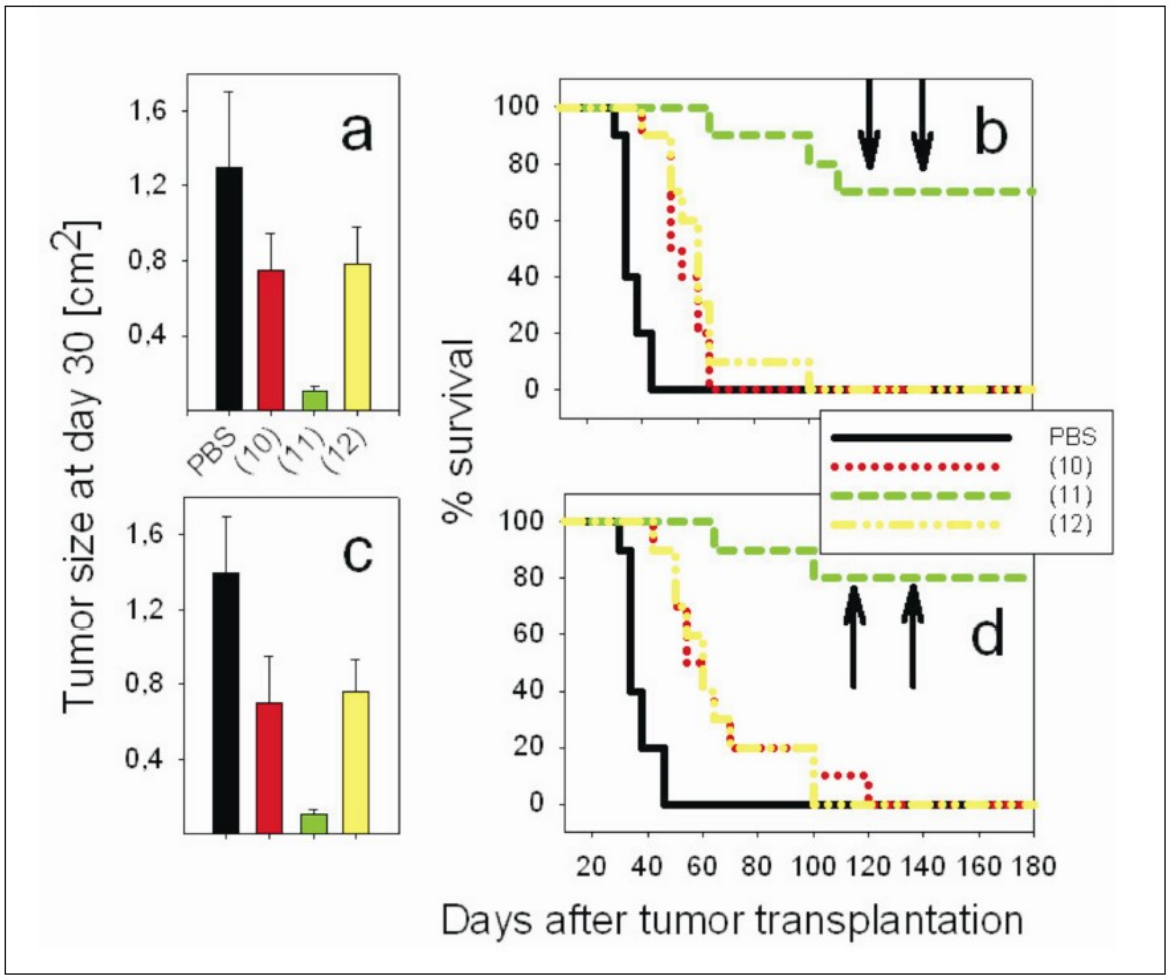

Figure 15. GlcNAc dimers of optimized linker length provide mice with protection against B16F10 melanoma[/46/]: (a) *tumor size at day 30 after injection.* ***(b)*** *survival of experimental mice bearing melanoma. Reinjection of 10^6 fresh melanoma cells at day 120 and 140 is indicated by an arrow.* ***(c)*** *and* ***(d)*** *identical experiments obtained for an optimized administration reginem for GlcNAc dimer. Compound (11) in these experiments was the one with linker length optimized at decyl.*

Only when the individual compounds are tested in complex biological systems such as *in vivo* experimental tumor therapy model, the ultimate knowledge about their effects may be obtained. However, since the *in vivo* tests are time consuming and expensive, very often we resort to simpler estimations using several *in vitro* tests, of which cellular activation test and assays of cytotoxicity and apoptosis have proved to be the most useful. Therefore, we performed activation assays based on monitoring of inositolphosphate production and intracellular Ca^{2+} level elevation, experiments regarding natural killing of leukemic cell line K562 by peripheral blood mononuclear cells isolated from healthy donors and activated using the tested compounds and apoptosis assays in which we observed hyperactivation of these cells and the resulting cytotoxicity-induced cell death.

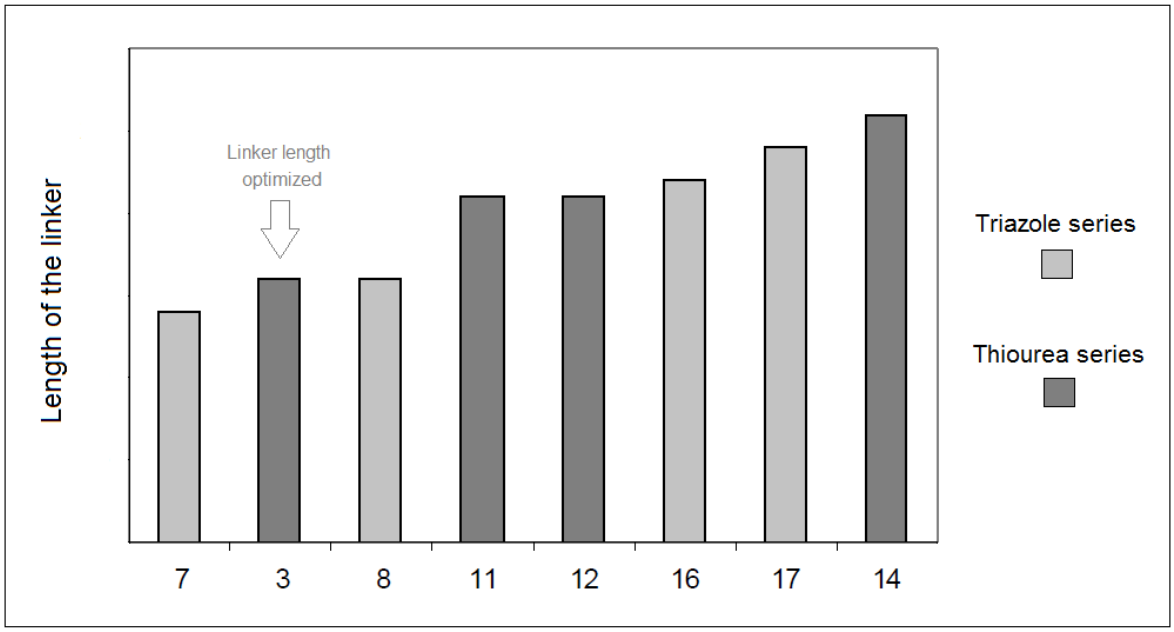

Figure 16. Arrangement of the tested compounds according to the length of the linker: *Linker length was estimated as a mutual distance of carbon 1 in the sugar units. The arrow indicates the compound in which the linker length was optimized from the point of view of increased activation and cross-linking of NKR-P1 receptor in mice in vivo models (compound* ***3****).*

In order to draw exact and correct conclusions about structure/activity relations, one will have to consider the three dimensional structure of individual compounds both in solution and after their interaction with the receptor. This is inherently very complicated, but even the rough estimations may be useful in some specific instances. In the structures tested here, we measure the mutual distance of

carbon 1 in the sugar units. On this basis, the tested compounds were lined up according to the length of the linker between two saccharide units. This arrangement is shown on figure 16 (page 46).

Considering this linker-length dependancy, we should assume that only compounds **3**, **7** and **8** are able to cross-link and activate NKR-P1 receptor. This was not entirely true in case of compound **3** that had no significant effect on precipitation of soluble NKR-P1 and only average effect on activation of PBMC. On the other hand, some precipitation (9 %) of soluble NKR-P1 was observed in case of compound **17** that scored best in activation assays, so as in cytotoxicity assays. Precipitation of soluble CD69 receptor was achieved by all compounds except compound **3** and **7**. The highest effect in CD69 precipitation assays was observed in case of compounds **16** and **17**. Although CD69 activation is not markedly dependent on linker length, the affinity of the ligand for the receptor is increased if a bulky slightly hydrophobic group is present in the proximity of saccharide unit. This is achieved in compounds **17** and **16**, to the less extent in compounds **11**, **12** and **14**.

Compound **17** proved its qualities as a potential high activity agent in tumor immunotherapies as it scored best in activation assays, natural killing assays and as it evoked almost none cytotoxicity-induced cell death. Since majority of the tested compounds had significant effects on activation of lymphocytes and natural killing of leukemic cell line, even though the effects on CICD were very low (compounds in triazole series), they are suitable for *in vivo* / *ex vivo* experiments on adequate animal model.

6. CONCLUSION

The aim of this work was to evaluate *in vitro* effect of several carbohydrate dimers used as ligand mimetics for receptors of NK cells on peripheral blood mononuclear cells. I performed cellular activation assays based on intracellular calcium monitoring and inositolphosphate production, assays of cytotoxicity and activation-induced cell death. The results we have obtained can be summarized as follows:

- All of the tested compounds except compound **7** and **8** affected phosphoinositide signaling in peripheral blood mononuclear cells. The highest effect on production of inositolphosphates was observed upon activation with compounds **17** and **11**.
- Similar results were obtained by monitoring intracellular Ca^{2+} levels. Although compounds **7** and **8** remained almost completely inactive, compound **17** caused the biggest Ca^{2+} elevation inside the cells.
- All of the tested compounds showed some increase in natural killing of the leukemic cell line K562, except compound **12** that showed no significant effect.
- Compounds of triazole series (**7**, **8**, **16** and **17**) did not induce CICD. On the other hand, compounds of thiourea series (**3**, **11**, **12** and **14**) enhanced activation-induced apoptosis.
- Compound **17** proved to be the most promising lead structure for *in vivo* testing and further development of more potent derivatives.

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Svoluji k zapůjčení této práce pro studijní účely a prosím, aby byla řádne vedena evidence vypůjčovatelů.

Jméno a příjmení, adresa	Číslo OP	Datum vypůjčení	Poznámka